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PRACTICAL
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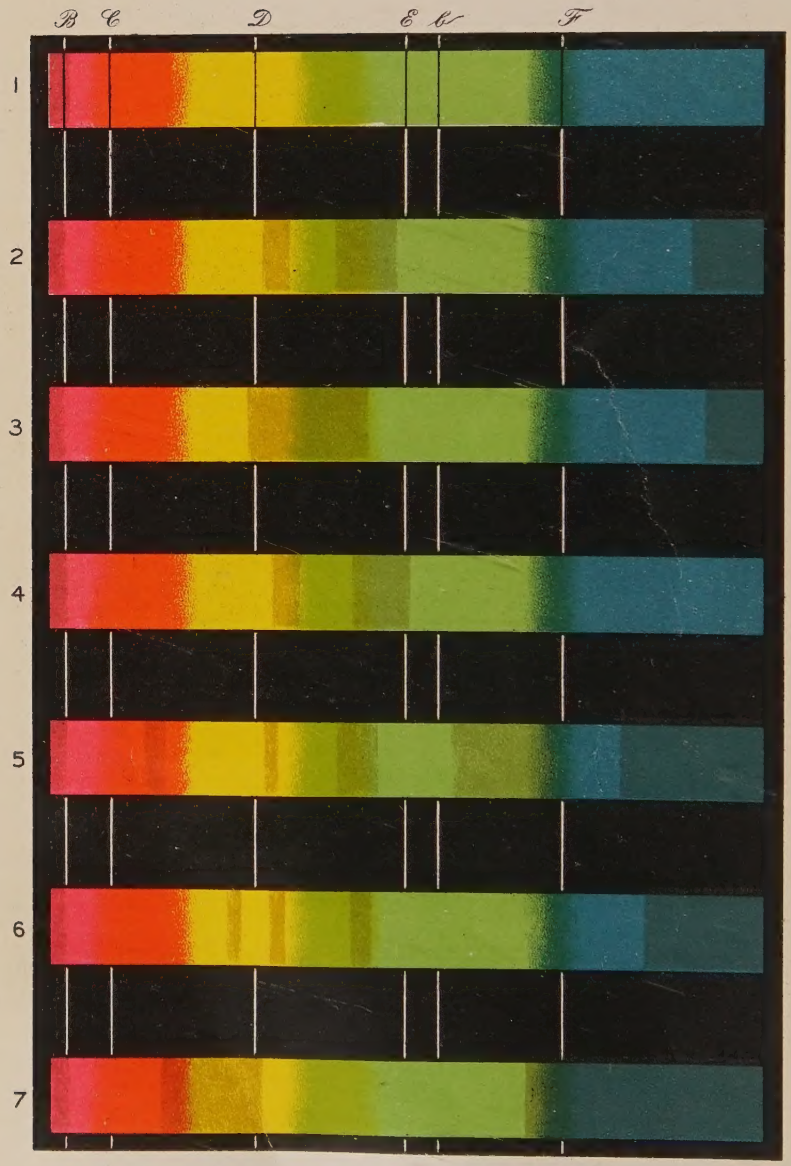
HAWK AND BERGEIM

Absorption Spectra.

PLATE I.



2 Oxyhaemoglobin.

3 Haemoglobin.

4 Carboxy-haemoglobin.

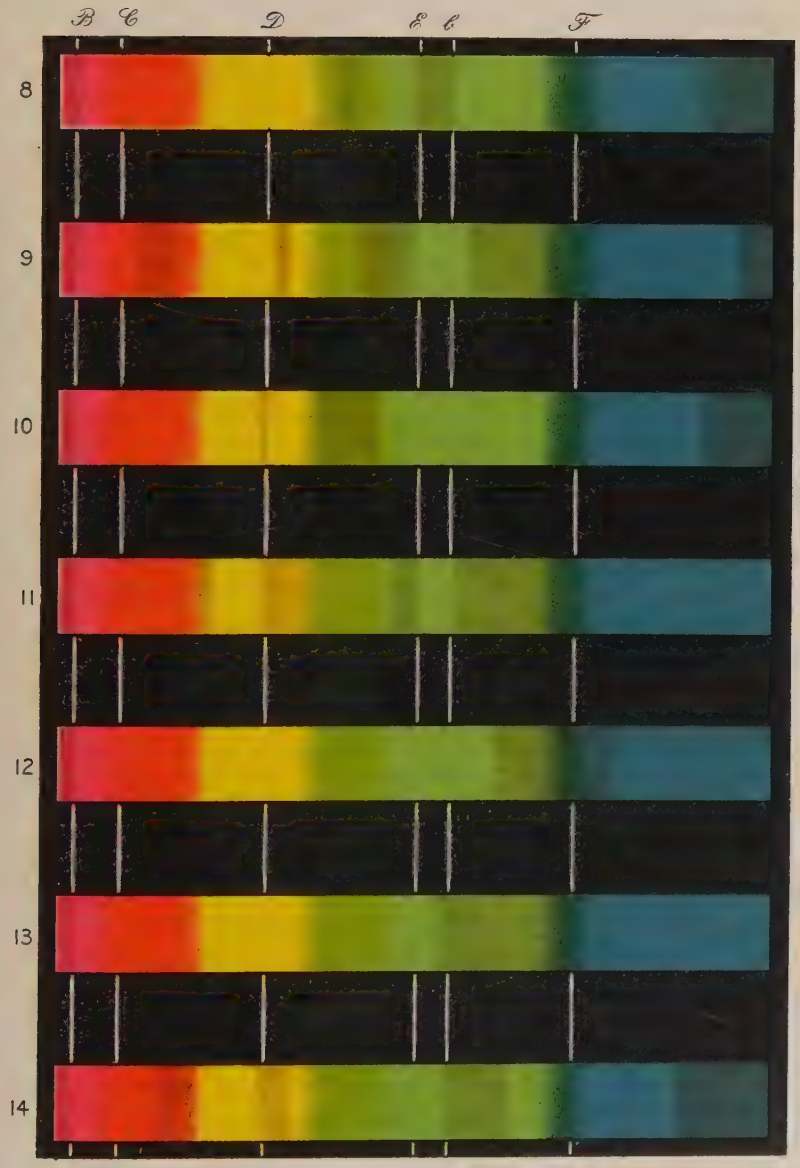
5 Neutral Met-haemoglobin.

6 Alkaline Met-haemoglobin.

7 Alkali Haematin.

Absorption Spectra.

PLATE II.



8 Reduced Alkali Haematin or Haemochromogen.

9 Acid Haematin in ethereal solution.

10 Acid Haematoporphyrin.

11 Alkaline Haematoporphyrin.

12 Urobilin or Hydrobilirubin in acid solution.

13 Urobilin or Hydrobilirubin in alkaline solution after the addition of zinc chloride solution.

14 Bilicyanin or Cholecyanin in alkaline solution.

PRACTICAL
PHYSIOLOGICAL CHEMISTRY

A BOOK DESIGNED FOR USE IN COURSES IN PRACTICAL PHYSIOLOGICAL CHEMISTRY IN SCHOOLS OF MEDICINE AND OF SCIENCE

BY

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NINTH EDITION, REVISED AND ENLARGED

WITH TWO FULL-PAGE PLATES OF ABSORPTION SPECTRA IN COLORS SIX ADDITIONAL FULL-PAGE COLOR PLATES AND TWO HUNDRED AND SEVENTY-THREE FIGURES OF WHICH TWELVE ARE IN COLORS

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PREFACE TO THE NINTH EDITION

It is with a feeling of distinct pleasure and satisfaction that I go before the friends of former editions of my "Practical Physiological Chemistry" with this volume which sets a standard of excellence considerably above that of any previous edition.

In addition to a most comprehensive and careful revision of the entire volume, entailing the complete rewriting of several of the more important chapters, it has been found necessary, in order to improve upon the previous high standard of the volume, to introduce several new chapters and sections. Therefore, notwithstanding the elimination of much subject matter from the previous edition, the volume has of necessity been increased in size. It has been my policy, throughout the life of this volume, to give to the student, the teacher, the physician and the investigator the very best in the way of methods, theories, conclusions, etc., which come to us as the result of the labors of the leading biochemists of the world. Because of the growing importance and widening scope of our science this task becomes more onerous with each revision.

It is not feasible to mention specifically each new feature which goes to make up the general excellence of the volume. However, a brief mention of the new chapters and sections will serve to indicate the character of some of the more important lines of revision.

Seven new chapters have been included in this new edition, four of them covering subjects not heretofore considered in this book. The new chapters are:

Chapter I:—"Physical Chemistry of True and Colloidal Solutions."

Chapter XIV:—"Absorption."

Chapter XV:—"Putrefaction and Detoxication."

Chapter XVIII:—"The Chemistry of the Blood. Tissue Analysis."

Chapter XIX:—"Respiratory Metabolism and Neutrality Regulation."

Chapter XX:—"The Endocrine Organs."

Chapter XXI:—"Energy Metabolism."

Chapter I includes understandable discussions of physicochemical procedures of importance in biochemistry.

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Chapter XIV contains a discussion of the intestinal absorption of the various food elements, together with newly elaborated experiments on the subject.

Chapter XV includes a review of the various types of putrefaction products and the chemistry involved in their detoxication in man and the lower animals. Among the experiments is given Bergeim's procedure for the estimation of intestinal reductions.

Chapter XVIII includes a revised table of normal variations of blood constituents with pathological changes, and a discussion of the importance of blood chemistry.

Chapter XIX includes a consideration of acid-base balance founded on the modern physico-chemical conception of the hydrogen ion concentration of buffer solutions and the physiological mechanisms brought into play for its regulation. Complete details of *manometric methods* of gas analysis, including urea in urine (unpublished to date) are also given.

Chapter XX contains a brief survey of the chemistry of the various hormones, with experiments on their preparation, determination and physiological effects. A hitherto unpublished illustration of adrenaline crystals is included.

Chapter XXI includes a survey of basal metabolism, embracing clinical interpretation, normal standards, methods and calculations.

In addition to these new chapters the revision has included the introduction of nine new sections of importance. These embrace:—

"Oxidation and Reduction Systems," included in Chapter II.

"Photosynthesis," included in Chapter III

"Amino acids," included in Chapter V.

"The Normal Gastric Response to Standard Foods," included in Chapter VIII.

"Dietary Deficiencies and Vitamins," including "Vitamin Assays" and "Color Reactions" in Chapter XXXII.

"Ultraviolet Radiation," included in Chapter XXXII.

"Rickets," included in Chapter XXXII.

"Ketosis," included in Chapter XXXII.

"Manipulatory Procedures" in Metabolism Experiments, including "Cowgill's Procedure for Metabolism Experiments on Dogs," included in Chapter XXXII.

It is hoped that the rearrangement, rewriting and expanding of the final chapter on *Studies in Metabolism* will make this chapter particularly useful to those interested in this important field of biochemistry.

While the modern trend toward physical chemistry is recognized by the material in Chapters I and XIX, the book, which was originally modelled after the courses given at Yale University and the College of Physicians and Surgeons of Columbia University, still maintains its former character.

An important feature of the revision has been the introduction of nearly one hundred new illustrations, including the reproduction of two new full page colored plates (Plate IV on the Shohl-King procedure for pH of gastric contents, and Plate VIII which reproduces the excellent figures published by Bills and McDonald on normal and rachitic bones). Furthermore, many of the old figures have been renewed from new drawings or photographs.

As in the past, an outstanding feature of the book is the comprehensive character and availability of its quantitative methods, which cover biochemical and clinical procedures. In certain instances several procedures have been suggested for the same purpose. This has been done to afford a desirable freedom of choice.

Throughout the book we have also made an effort to give references to the most recent articles of importance, laying particular emphasis on review articles. We feel that these references are of importance not alone to the teacher, physician and investigator, but to the student as well.

It is manifestly impossible for any single laboratory course in biochemistry to cover the mass of material included between the covers of this book. It is within the province of the teacher to select such portions as suit his particular needs. Because of the comprehensive treatment given the major fields of biochemistry, the book will be found to fit in nicely in courses of widely divergent character, e.g., scientific, medical, dental, agricultural, pharmaceutical, household science, etc.

Chapters I and XIV, were written by Professor Olaf Bergeim of the University of Illinois, College of Medicine; Chapter XV, by Professor Carl P. Sherwin, Head of the Department of Physiological Chemistry of Fordham University; Chapters XVIII and XIX, by Bernard L. Oser, of the Food Research Laboratories, Inc., New York City, (formerly of the staff of the Philadelphia General Hospital); Chapter XX, by Dr. Edward Larson, of the University of Illinois, College of Medicine, and Chapter XXI, by Dr. Cameron V. Bailey, of the New York Post Graduate Medical School and Hospital. The section on "Amino Acids" was written by Professor Sherwin; the section on "Oxidation and Reduction Systems," by Professor Bergeim; the section on "Ultraviolet Radiation" was prepared by Dr. H. S. Mayerson, of the Depart-

ment of Physiology, Yale University, School of Medicine; the section on "Rickets" by Dr. T. F. Zucker, of the Department of Pathology of the College of Physicians and Surgeons, New York City (Columbia); the section on "Ketosis" by Dr. Harold Levine, of the Food Research Laboratories, Inc., New York City (formerly of the Department of Physiological Chemistry of Yale University); and the section on "Photosynthesis," by Mr. Arthur G. Cole, of the University of Illinois, College of Medicine.

The writer is deeply obligated to the above scientists and physicians for their hearty and self-sacrificing cooperation which has materially added to the excellence of the present volume.

To Professor Olaf Bergeim and Bernard L. Oser, the writer owes a special debt of gratitude. Professor Bergeim has played an important part in the revision of several editions of this volume and in recognition of this fact, together with the high regard I have for him both personally and professionally, it has been a pleasure to make him co-author of the present edition.

The services of my colleague, Bernard L. Oser, have been particularly valuable. In addition to assuming a major role in the general revision of the volume, he was able, on the basis of a wide experience at the Philadelphia General Hospital, to rewrite two chapters of great scientific and clinical importance ("Blood Analysis" and "Respiratory Metabolism") and bring them strictly up to date.

The authors are indebted to Professor Lafayette B. Mendel for advice looking to the betterment of the book; to Professor William H. Welker, for suggestions, especially with reference to urinary and blood proteins and for his courtesy in furnishing photographs of crystals of horse serum albumin, Bence-Jones protein and the protein of Hektoen, Kretschmer and Welker; to Professor Victor C. Myers, for many useful suggestions in the field of blood and urine analysis, and for a photograph of his bicolorimeter; to Professor Stanley R. Benedict, for a critique of methods, expecially those involving the determination of sugar and uric acid; to Professor D. Wright Wilson, for a cut illustrating the influence of exercise on the composition of the urine; to Professor Arthur H. Smith, for permission to illustrate his cages for albino rats; to Professor F. C. Koch, for helpful suggestions; to Dr. Walter M. Boothby and Dr. Irene Sandiford, for permission to reproduce their "surface area nomogram"; to Professor H. S. Schwenk, for revision of the "scheme" for detection of carbohydrates (page 103); to Dr. Eugene F. Du Bois, for permission to insert his "surface area chart;" to Professor Raymond L. Stehle, for furnishing details of his urea method before publication; to Dr. Francis B. Kingsbury, for his aid in the introduction of the methods

for albumin and sugar adopted by the "Committee on Urinary Impairments of the Association of Life Insurance Medical Directors of America;" to Professor James B. Sumner, for permission to reproduce a cut of his crystalline urease; to Professor Edward C. Kendall, for permission to reproduce figures illustrating thyroxin and its action, including a photograph of his crystalline thyroxin; to Professor Francis G. Benedict, for a drawing of his "Oxycalorimeter;" to Professor J. B. Collip, for a photograph of the modified Parnas-Wagner distillation apparatus; to Professor Henry C. Sherman, for figures used in illustrating the subjects of "vitamin storage," "cystine deficiency" and "calcium deficiency;" to Professor V. G. Heller, for permission to use unpublished material on vitamin color reactions; to Dr. Donald D. Van Slyke, for permission to insert an unpublished method for urea in urine and to reproduce various charts and figures; to Drs. Alfred R. Shohl and John H. King, for the privilege of reproducing a colored plate used in the determination of the pH of gastric contents; to Mr. Arthur G. Cole, for preparing and photographing crystals of adrenaline, egg albumin and hemochromogen; to Dr. Charles E. Bills, for permission to reproduce Plate VIII, representing the results of the "Line Test" on normal and rachitic bones; to Dr. G. L. Keenan, for permission to reproduce his figures of crystalline amino acids; to Dr. M. H. Power (Mayo Clinic), for permission to insert an unpublished sugar method; to Dr. Francis W. Power (Fordham) for an original drawing of a rotary extractor (Fig. 85); to Dr. Weston A. Price, for permission to reproduce a figure illustrating the action of ultraviolet light; to Dr. W. G. Karr, for permission to use two figures illustrating the effect of diet and of fasting on carbohydrate utilization; to Dr. George R. Cowgill, for suggestions regarding his "Metabolism Procedure;" to Mr. Francis H. Parker, for permission to insert an unpublished method for indican; to Dr. J. K. Marcus, for assistance on vitamin color reactions; to Dr. O. H. Gaebler, for useful suggestions and a drawing of his continuous extraction apparatus; to Dr. Cyrus H. Fiske, for methods and permission to reproduce a figure of his filtration tube; to Dr. W. H. Griffith, for permission to insert an unpublished method for hippuric acid; to Dr. Harold Levine, for permission to reproduce figures of his metabolism cage and food dish; and to Dr. Benjamin Harrow, for useful criticism.

The authors are also indebted to Mrs. Philip B. Hawk, for the preparation of an excellent revised index, to Miss Mona Oser, for assistance in proof reading, to the Arthur H. Thomas Co., for numerous cuts, and to various Journals, notably the *Journal of Biological Chemistry; Journal of the American Chemical Society; Journal of the American Medical Association; American Journal of the Medical Sciences; British*

Medical Journal; Lancet; Boston Medical and Surgical Journal; Journal of Laboratory and Clinical Medicine; Biochemical Journal; American Journal of Physiology; Industrial and Engineering Chemistry; and the *Bulletin of the Johns Hopkins Hospital*, for the privilege of using copyrighted material.

It is a pleasure once more to attest to the unfailing courtesy of the publishers, P. Blakiston's Son and Co., and to the fine spirit of cooperation of the Maple Press.

PHILIP B. HAWK.

NEW YORK CITY
October, 1st, 1926,

CONTENTS

CHAPTER I

PAGE

PHYSICAL CHEMISTRY OF TRUE AND COLLOIDAL SOLUTIONS 1

CHAPTER II

ENZYMES AND THEIR ACTION. 41

CHAPTER III

CARBOHYDRATES. 66

CHAPTER IV

SALIVARY DIGESTION. 104

CHAPTER V

PROTEINS: THEIR DECOMPOSITION AND SYNTHESIS. AMINO ACIDS 113

CHAPTER VI

PROTEINS: THEIR CLASSIFICATION AND PROPERTIES. 157

CHAPTER VII

NUCLEIC ACIDS AND NUCLEOPROTEINS. 188

CHAPTER VIII

GASTRIC DIGESTION. 200

CHAPTER IX

GASTRIC ANALYSIS. 217

CHAPTER X

FATS. 245

CHAPTER XI

PANCREATIC DIGESTION 255

CHAPTER XII

INTESTINAL DIGESTION. 266

CHAPTER XIII

BILE. 273

CHAPTER XIV

ABSORPTION. 283

CHAPTER XV

PUTREFACTION AND DETOXICATION 291

CHAPTER XVI

Page

Feces 308

CHAPTER XVII

Blood and Lymph 331

CHAPTER XVIII

The Chemistry of the Blood. Tissue Analysis 356

CHAPTER XIX

Respiratory Metabolism and Neutrality Regulation. 419

CHAPTER XX

The Endocrine Organs. 473

CHAPTER XXI

Energy Metabolism 491

CHAPTER XXII

Milk 530

CHAPTER XXIII

Epithelial and Connective Tissues: Teeth. 549

CHAPTER XXIV

Muscular Tissue 559

CHAPTER XXV

Nervous Tissue 575

CHAPTER XXVI

Urine: General Characteristics of Normal and Pathological Urine. . . . 581

CHAPTER XXVII

Urine: Physiological Constituents. 592

CHAPTER XXVIII

Urine: Pathological Constituents. 637

CHAPTER XXIX

Urine: Organized and Unorganized Sediments. 680

CHAPTER XXX

Urine: Calculi 698

CHAPTER XXXI

Urine: Quantitative Analysis 702

CHAPTER XXXII

Studies in Metabolism. Dietary Deficiencies; Vitamin Assays; Ultraviolet Irradiation; etc. 789

Appendix

Reagents and Solutions. 882

Tables for Respiration Experiments. 894–902

Logarithm Tables 903–904

Atomic Weights 905

Index 907

LIST OF ILLUSTRATIONS

PLATE

I. Absorption Spectra
II. Absorption Spectra . . . . . *Frontispiece*

III. Osazones . . . . . Opposite page 72
IV. Color Chart for pH of Gastric Contents . . . . . Opposite page 233
V. Normal Erythrocytes and Leucocytes . . . . . Opposite page 335
VI. Uric Acid Crystals . . . . . Opposite page 602
VII. Ammonium Urate . . . . . Opposite page 685
VIII. Line Test on Normal and Rachitic Tibias . . . . . Between pages 838 and 839

FIGURE PAGE

1. Hydrogen Ion Concentration Chart 12
2. Ostwald Viscosimeter 17
3. Stalagmometer 22
4. Du Noüy Tensiometer 23
5. Comparator Block 24
6. Diagram Showing Relations at Metal Surface in Solution Containing Its Ions . 28
7. Connections for Electrometric Determination of Hydrogen Ion Concentration . . 31
8. Titration Curves of Various Acids 33
9. Connections for Double Hydrogen Electrode System 34
10. Curves of Buffer Mixtures 35
11. Connections for Determination of Electrical Conductivity 39
12. Conductivity Cells 39
12A. Urease Crystals 46
13. Diagram of Microrespirometer 51
14. Vacuum Tube (Thunberg) 62
15. Apparatus for Quantitative Determination of Catalase 63
16. Dialyzing Apparatus for Students' Use 74
17. Apparatus for Alcoholic Fermentation Experiment 78
18. Iodoform 78
19. Einhorn Saccharometer 79
20. One Form of Laurent Polariscope 80
21. Diagrammatic Representation of the Course of the Light through the Laurent Polariscope 81
22. Polariscope (Schmidt and Hänsch Model) 82
23. Potato Starch 94
24. Bean Starch 94
25. Arrowroot Starch 94
26. Rye Starch 94
27. Barley Starch 94
28. Oat Starch 94
29. Buckwheat Starch 94
30. Maize Starch 94
31. Rice Starch 94
32. Pea Starch 94
33. Wheat Starch 94
34. Microscopical Constituents of Saliva 108
35. Glycocoll (Synthetic) 130

FIGURE PAGE
36. Alanine . . . 131
37. Serine . . . 132
38. Leucine . . . 134
39. Aspartic Acid . . . 136
40. Glutamic Acid . . . 139
41. Levo-α-Proline . . . 140
42. Copper Salt of Proline . . . 141
43. Cystine . . . 143
44. Lysine Picrate . . . 146
45. Phenylalanine . . . 147
46. Tyrosine . . . 148
47. Histidine Dihydrochloride . . . 151
48. Tryptophane . . . 154
49. Coagulation Temperature Apparatus . . . 170
50. Crystallized Egg Albumin . . . 171
51. Horse Serum Albumin . . . 171
52. Edestin . . . 174
53. Excelsin, the Protein of Brazil Nut . . . 175
54. Guanine Chloride . . . 197
55. Relationships of the Normal Evacuation Times of Standard Foods . . . 209
56. The Evacuation Times of Fast and Slow Stomachs . . . 209
57. Evacuation Times of Standard Foods . . . 209
58. Curves Showing Stimulatory Power of Water . . . 210
59. Curves Showing Stimulatory Power of Water . . . 210
60. Curves Showing Stimulatory Power of Beef Extract . . . 214
61. Curves Showing Psychical Stimulation of Gastric Secretion . . . 215
62. Normal and Pathological Curves after an Ewald Meal . . . 217
63. Rehfuss Stomach Tube . . . 218
64. Ryle's Gastric Tube . . . 219
65. Bergeim Intragastric Conductance Apparatus . . . 219
66. Curves Showing Relationship of Conductance to Acidities . . . 219
67. Influence of Acid Introduced into the Normal Human Stomach . . . 220
68. Showing Gastric Response of Same Individual on Four Different Occasions . . . 227
69. Acidity Curves of Normal Human Stomach . . . 230
70. Acidity Curves from a Case of Hypersecretion . . . 231
71. Acidity and Protein Curves in Gastric Carcinoma . . . 232
72. Total Acidity and Protein Curves in Benign Achylia . . . 232
73. Microscopical Constituents of the Gastric Contents . . . 241
74. Beef Fat . . . 245
75. Mutton Fat . . . 248
76. Pork Fat . . . 251
77. Palmitic Acid . . . 252
78. Melting-point Apparatus . . . 253
79. Bile Salts . . . 276
80. Bilirubin (Hematoidin) . . . 276
81. Cholesterol . . . 281
82. Taurine . . . 282
83. Glycocoll . . . 282
84. Showing Absorption of Ca and P from Different Parts of the Intestinal Tract of Rachitic Rats . . . 289
85. Rotary Extractor . . . 305
86. Hematoidin Crystals from Acholic Stools . . . 309
87. Charcot-Leyden Crystals . . . 312
88. Boas' Sieve . . . 316

FIGURE PAGE
89–94. Microscopical Constituents of Feces 317–318
95. Oxyhemoglobin Crystals from Blood of the Guinea-pig 337
96. Oxyhemoglobin Crystals from Blood of the Rat 337
97. Oxyhemoglobin Crystals from Blood of the Horse 337
98. Oxyhemoglobin Crystals from Blood of the Squirrel 337
99. Oxyhemoglobin Crystals from Blood of the Dog 338
100. Oxyhemoglobin Crystals from Blood of the Cat 338
101. Oxyhemoglobin Crystals from Blood of the Necturus 338
102. Effect of Water on Erythrocytes 346
103. Hemin Crystals from Human Blood 350
104. Hemin Crystals from Sheep Blood 350
105. Sodium Chloride 351
106. Hemochromogen Crystals 352
107. Myers Test-tube Colorimeter 360
108. Hellige-Leitz Wedge Colorimeter 360
109. Bock Benedict Colorimeter 361
110. Spencer-Duboscq Colorimeter 361
111. Triple Row Comparator Block 361
112. Myers' Bicolorimeter 362
113. Bloor's Nephelometer 364
114. Nephelometer in Position, Showing Relation to Source of Light 365
115. Diluting Pipette 367
116. Apparatus for Distillation of Ammonia from Urine 371
117. Distillation Apparatus Used in Clark-Collip Urea Method 372
118. Aspiration Apparatus for Urea Determination 374
119. Folin-Wu Sugar Tube 381
120. Sugar Tolerance Curves 389
121. Blood Sugar Curve of Diabetic after Glucose Ingestion 390
122–123. Extraction Apparatus for Cholesterol Determination 392
124. Direct-vision Spectroscope 414
125. Angular-vision Spectrometer 415
126. Diagram of Angular-vision Spectroscope 415
127. Titration Curve for H_2CO_3: $BHCO_3$ System 421
128. Normal and Abnormal Variations of Acid-base Balance in Oxygenated Human Whole Blood 427
129. Normal and Abnormal Variations of the $BHCO_3$ and pH in Serum or Oxalated Plasma 428
130. Van Slyke Carbon Dioxide Apparatus 432
131. Tube Used in Collecting Blood 432
132. Precision Model of Van Slyke Apparatus 433
133. Separatory Funnel Used in Saturating Blood Plasma with Alveolar Air 434
134. Fridericia Apparatus 442
135. Van Slyke and Neill Manometric Gas Analysis Apparatus 459
136. Open Type of Manometric Apparatus 460
137. Closed Type of Manometer Tube 461
138. Extraction Chamber 462
139. Operation of Delivery Pipette 462
140. Use of Calcium Chloride Tube Reservoir 464
141. Bulb for Preparing and Storing Gas-free Solutions 464
142. Nomogram for Calculation of Plasma CO_2 Capacity 466
143. Schematic Chart of Endocrine System 473
144. Adrenaline (Ephinephrine) 475
145. Effect of Thyroxin on Growth of a Cretin 480
146. Effect of Thyroxin on Myxedema 480

FIGURE PAGE
147. Thyroxin . . . 481
148. Inspired and Expired Air . . . 499
149. Surface Area Nomogram . . . 505
150. DuBois Surface Area Chart . . . 506
151. Form Sheet for Study of Respiratory Metabolism . . . 508
152. Diagram of the Cot Chamber of Dr. F. G. Benedict . . . 511
153. Diagram of the Benedict-Roth Respiration Apparatus . . . 512
154. Benedict-Roth Respiration Apparatus with Kymograph . . . 513
155. Graphic Record of a Metabolism Test . . . 514
156. Benedict's Student Model Respiration Apparatus . . . 516
157. Diagram of Gasometer for Collection of Expired Air . . . 519
158. Bailey Gasometer . . . 520
159. Head Mask . . . 521
160. Illustrating Manner of Operating Bailey Gas Sampling Bottles . . . 522
161. Bailey's Modification of the Haldane-Henderson Gas Analysis Apparatus . . . 523
162. Diagram of Bailey's Modification of the Haldane-Henderson Gas Analysis Apparatus . . . 524
163. Efficiency of Milk in the Diet . . . 530
164. Normal Milk and Colostrum . . . 532
165. Curd of Human Milk . . . 533
166. Curd of Human Milk . . . 533
167. Curd of Cows' Milk . . . 534
168. Curd of Cows' Milk . . . 534
169. Lactose . . . 537
170. Calcium Phosphate . . . 542
171. Centrifuge Tube Used in Babcock Fat Method . . . 544
172. Croll's Fat Apparatus . . . 545
173. Soxhlet Apparatus . . . 546
174. Creatine . . . 564
175. Xanthine . . . 566
176. Hypoxanthine Silver Nitrate . . . 573
177. Hypoxanthine Chloride . . . 573
178. Xanthine Silver Nitrate . . . 573
179. Diagram Illustrating the Secretion of Urine . . . 581
180. Deposit in Ammoniacal Fermentation . . . 585
181. Deposit in Acid Fermentation . . . 586
182. Urinometer and Cylinder . . . 587
183. Beckmann-Heidenhain Freezing-point Apparatus . . . 588
184. Urea . . . 595
185. Urea Nitrate . . . 598
186. Melting-point Tubes Fastened to Bulb of Thermometer . . . 599
187. Urea Oxalate . . . 600
188. Pure Uric Acid . . . 604
189. Creatinine . . . 606
190. Creatinine-Zinc Chloride . . . 608
191. Hippuric Acid . . . 613
192. Allantoin from Cat's Urine . . . 616
193. Benzoic Acid . . . 620
194. Calcium Sulphate . . . 630
195. "Triple Phosphate" . . . 633
196. Albumoscope . . . 646
197. Crystals of Bence-Jones Protein . . . 648
198. Protein of Hektoen, Kretschmer and Welker . . . 649
199. Pentosazone . . . 663

FIGURE PAGE
200. Marsh Apparatus . . . 669
201. The Electric Centrifuge . . . 680
202. Conical Centrifuge Tube . . . 680
203. Calcium Oxalate . . . 682
204. Calcium Carbonate . . . 682
205. Various Forms of Uric Acid . . . 684
206. Acid Sodium Urate . . . 685
207. Cystine . . . 685
208. Crystals of Impure Leucine . . . 686
209. Epithelium from Different Areas of the Urinary Tract . . . 689
210. Pus Corpuscles . . . 690
211. Hyaline Casts . . . 691
212. Granular Casts . . . 692
213. Granular Casts . . . 693
214. Epithelial Casts . . . 693
215. Blood, Pus, Hyaline and Epithelial Casts . . . 693
216. Fatty Casts . . . 694
217. Fatty and Waxy Casts . . . 694
218. Cylindroids . . . 695
219. Crenated Erythrocytes . . . 696
220. Human Spermatozoa . . . 696
221. Folin Fume Absorber . . . 711
222. Folin Wright Distillation Apparatus . . . 713
223, 224. Forms of Aeration Apparatus (Folin and Farmer) . . . 715
225. Bock and Benedict Distillation Apparatus . . . 716
226. Van Slyke and Cullen Aeration Apparatus . . . 720
227. Folin's Ammonia Aeration Apparatus . . . 725
228. Folin Improved Absorption Tube . . . 725
229. Continuous Extraction Apparatus (Griffith) . . . 742
230. Esbach's Albuminometer . . . 755
231. Continuous Extraction Apparatus (Gaebler) . . . 769
232. Filtration Tube for Sulphur Determination . . . 775
233. Rat Cage for Nutrition Experiments, Assembled . . . 793
234. Rate Cage for Nutrition Experiments, Showing Individual Parts . . . 793
235. Food Dish . . . 794
236. Rat Cage for Stock Animals . . . 795
237. Activity Cage . . . 795
238. Metabolism Cage for Small Animals . . . 795
239. Cross-section of Metabolism Cage for Small Animals . . . 796
240. Food Consumption Chart . . . 797
241. Data Sheet for Vitamin Assay . . . 797
242. Growth Curve Chart . . . 798
243. Effects of Moderate and of Increasingly Intensive Feeding of Vitamin A upon Storage . . . 806
244. Effects of Intensive Feeding of Vitamin A upon Storage . . . 807
245. Guinea Pig with Scurvy, Showing "Scurvy Position" . . . 817
246. Guinea Pig with Scurvy, Showing "Face Ache Position" . . . 817
247. Irradiation of Albino Rats . . . 827
248. Effect of Ultraviolet Light on Leg-weakness in Chicks . . . 829
249. Growth Curve of Albino Rat . . . 832
250. Growth Curve of Albino Rat Showing Importance of Vitamin "B" . . . 833
251. Rat Fed a Diet Containing Sufficient Vitamin "B" . . . 834
252. Rat Fed a Diet Deficient in Vitamin "B" . . . 834
253. Growth Curve of Albino Rat Showing Importance of Vitamin "B" . . . 835

FIGURE PAGE
254. Rat Fed a Diet Deficient in Vitamin A and Rat Fed an Adequate Diet 836
255. Vitamine A and Growth. 836
256. Active Rickets (Radiograph). 837
257. Healed Rickets (Radiograph). 837
258. Showing Importance of Adequate Protein (Amino Acids) in the Diet 839
259. Curve Showing Influence of a Deficiency of Cystine in the Diet. 840
260. Quantitative Relationship of Cystine to Growth 841
261. Curve Showing Influence of a Deficiency of Lysine in the Diet 843
262. Rat Receiving Wheat Protein and Gelatin. The Diet Contained Sufficient Lysine. 844
263. Rat Receiving Wheat Protein Only. This Diet was Deficient in Lysine 844
264. Growth Curve of Rat with and without Calcium and Phosphorus in the Diet 846
265. Skeletons of Twin Albino Rats Showing Influence of Calcium Deficiency. . . . 847
266. Berthelot-Atwater Bomb Calorimeter 852
267. Oxycalorimeter of Benedict and Fox. 855
268. Influence of Protein Ingestion on Endogenous Uric Acid Output 858
269. The Endogenous Uric Acid Output during Fasting 858
270. Blood Sugar as Influenced by Diet 863
271. Influence of Fasting on Carbohydrate Utilization 864
272. Influence of Diet on Carbohydrate Utilization 865
273. Curve Showing Effect of Exercise on the Composition of the Urine 867

PHYSIOLOGICAL CHEMISTRY

CHAPTER I

PHYSICAL CHEMISTRY OF TRUE AND COLLOIDAL SOLUTIONS[1]

THE COLLOIDAL STATE

In ordinary, true solutions, the molecules of solute are dispersed in the solvent and, in electrolytes, these molecules are in equilibrium with their ions. The same substance may, however, exist in solution not only as molecules, or ions, but also as molecular aggregates. Thus, as von Weimarn[2] has shown, varying the concentrations of solutions of $Ba[CNS]_2$ and $MnSO_4$ from N/20000 to 7N causes a variation in form of the $BaSO_4$ precipitated from large crystals to a colloidal gel. From this and similar experiments, we may infer that in all precipitations, the process is one of growth from the ionic and molecular stage to the coarse precipitate, which involves the coalescing of these molecules to form molecular aggregates of increasing size. These aggregates may contain from two to millions of molecules and when the aggregate reaches a definite size, it separates out of solution as a precipitate. The particles, which are visible to the naked eye or under an ordinary microscope and which separate out from the solution on standing, are said to be in suspension. Particles of intermediate size from $200\mu\mu$, about the limit of microscopic vision, down to $1\mu\mu$ (1 millionth mm.), or molecular dimensions, are said to be in colloidal solution.

The differences in properties between colloidal and molecular solutions must, therefore, depend upon differences in the size of the particles. Colloidal particles in contrast with average molecules are large enough to separate the molecules of the solvent beyond the range of their cohesional attractions. They thus possess surface, and phenomena of *surface tension* come into play at the interfaces. The

[1] Bogue: Colloidal Behavior, Theory and Application, McGraw-Hill Book Co., New York, 1924.
Alexander: Colloid Chemistry, Theoretical and Applied, The Chemical Catalog Co., New York, 1926.
Loeb: Proteins and the Theory of Colloidal Behavior, 2nd Ed., McGraw-Hill Book Co., New York, 1924.

[2] von Weimarn: Die Allgemeinheit des Kolloidzustandes, Dresden and Leipzig, 1925.

extent of the forces here involved becomes clear when we realize that if a bit of any substance be subdivided until the particles are of colloidal dimensions, the surface area may be increased a million-fold. Substances which decrease surface energy, such as soaps, will tend to accumulate at the surface, and substances such as sodium chloride which increase surface tension will tend to be less concentrated there.

As the particles in a colloidal solution may have thousands of times the volume of those in the molecular solution, of two such solutions containing the same amount of substances by weight, the colloidal solution will contain far fewer particles. Certain physical properties of solutions depend not upon the nature, but upon the number of particles of solute in solution. Among these are *vapor pressure* and *osmotic pressure*. Colloidal systems, therefore, have very little effect in raising the boiling point or lowering the freezing point of solutions and exert only the slightest osmotic pressure.

Because of the size of the colloidal particles they do not pass through collodion, parchment or other membranes which are quite permeable to most substances in the molecular state. Thus by *dialysis* it is possible to separate colloids from substances which form molecular solutions. It was at one time thought possible to classify substances as crystalloids and colloids on the basis of dialysis and crystal formation. However, many substances may exist in either the crystalloid or colloid state depending upon conditions. Examples are barium sulphate, already mentioned, and certain proteins.

Colloidal particles are not visible to the naked eye. If, however, a colloidal solution be illuminated by a very intense light such as may be obtained from a lamp equipped with lenses which concentrate the light to a point, light will be scattered by the particles and become visible as a haze like that noted when sunlight passes through a dusty atmosphere. This is called the *Tyndall effect*. The reflected light from the particles if examined through a Nicol prism will be found to be polarized.

In a similar manner if certain colloidal solutions be examined under a microscope and the field be intensely illuminated from the side, instead of from the bottom, points of light corresponding to the particles will be seen. These points of light will be found to dance violently about a fixed point first in one direction and then in another. This phenomenon is called the *Brownian movement* and is due to bombardment of the particles by the molecules of the solvent. A microscope equipped in the above manner is called an *ultramicroscope*.

Colloidal particles are kept in suspension in part by their Brownian movement. Most colloidal particles also carry *electrical charges*. Since in a given colloidal solution the particles will possess similar charges,

they will repel each other and will not combine to form larger particles. If the solution further contains some substance such as gelatin which forms a protective film about the particles the tendency of the latter to combine is lessened. Any influence tending to prevent aggregation will increase stability.

Several methods may be used for the preparation of colloidal solutions. Colloidal particles have been prepared by simple grinding in a ball mill. More satisfactory is the colloid mill which operates at a very high speed. If two metal electrodes be brought close together under water and current passed so as to produce an arc, metal will be vaporized and will condense into particles of colloidal size. Thus colloidal gold or platinum may be prepared. A dilute solution of arsenious oxide in water if treated with hydrogen sulphide will undergo double decomposition with the formation of colloidal arsenious sulphide. If a very dilute solution of ferric chloride be boiled colloidal ferric hydroxide may be formed. The process is one of hydrolytic dissociation. Reduction may also be employed. Thus if a dilute solution of gold chloride be treated with formaldehyde under proper conditions the gold is reduced to the metallic form and the molecules aggregate to produce particles of colloidal size.

Colloidal solutions of the types just mentioned which contain particles of pure solid substances in a dispersed form are called *suspensoids*. Such solutions have little greater *viscosity* than the pure solvent. They are precipitated by small amounts of electrolytes because some of the charges on the particles which cause them to repel each other are neutralized by ionic charges and aggregation is permitted. When precipitated in this way or by evaporation of their solutions they will not go back into the colloidal state on adding more solvent, and are therefore said to be irreversible.

Much more important from the biological standpoint than the suspensoid state is the *emulsoid state*. Emulsoids include suspensions in water of proteins, starch, glycogen, agar, soaps, and silicic acid. In all of these cases the dispersed phase contains more or less water. Emulsoids are not precipitated by small amounts of electrolytes and are reversible. They possess a high viscosity, but a decreased surface tension. They are precipitated by the addition of large amounts of salts. If we dissolve about one per cent of gelatin, starch or soap in hot water and then allow to cool, translucent solids or jellies result. Such solutions are called *gels* to distinguish them from colloidal solutions not possessing this consistency and which are designated as *sols*. Where water is the dispersing medium the solutions are called *hydrogels* and *hydrosols* respectively. The taking up by emulsoid particles of

increasing amounts of water is called *imbibition*. It is essentially the same as the absorption of water by a gel with consequent swelling. In swelling heat is given off and very considerable pressures may be developed. Acidity increases the swelling of gels. Gels evidently possess some kind of a regular structure. Possibly they consist in some cases of a spongelike network of the less hydrated phase surrounding drops of the more hydrated material.

In the case of oil-water emulsions, either the oil or the water, may be the continuous phase. If the water be continuous in a medium of this type, only substances soluble in water may pass through. If the oil be continuous, only substances soluble therein may permeate.

Substances which reduce surface tension tend to accumulate in the surface films about the particles of a colloidal suspension. If they are soluble in the particles they are absorbed by them. If they are insoluble they will accumulate at the surfaces and the process is said to be one of *adsorption*. A charcoal suspension for example has the property of adsorbing many gases, liquids and solids. Adsorption is however very dependent upon the nature of the substances concerned. Negative colloids adsorb positive particles and vice versa. Thus the negative arsenious sulphide is most readily adsorbed by the positive ferric hydroxide. It has further been suggested that the adsorbed substance is generally held in loose chemical combination with the surface molecules of the particles through residual valences existing there. Orientation of the adsorbed molecules is also believed to occur. Thus when oleic acid is adsorbed by oil particles from a water solution the "polar" carboxyl group is directed outward from the particle into the water which more nearly resembles it in structure, while the "non-polar" alkyl radical is held within the oil particle.

The British physicist Donnan has made a contribution to our knowledge of the influence of ions in colloidal systems of the utmost importance in physiology. Let us suppose a membrane (indicated below by a vertical line) to be impermeable to the anion R of a salt NaR in solution on one side of the membrane but permeable to all other ions and salts involved. Let us further suppose that we have at the beginning of the experiment NaR on one side of the membrane and NaCl on the other. (See diagram at the left below.) NaCl will diffuse from 2 to 1 until at equilibrium conditions will be as indicated on the right.

Na^+	Na^+	Na^+	Na^+
R^-	Cl^-	R^-	
[1]	[2]	Cl^-	Cl^-
		[1]	[2]

Now from thermodynamic considerations it can be calculated, and by actual determination it can be shown, that at equilibrium the product of the concentrations of a pair of diffusible ions (in this case Na^+ and Cl^-) on one side of the membrane will be the same as the product of the concentrations of the same ions on the other side of the membrane. That is,[1]

$$[Na^+]_1\,[Cl^-]_1 = [Na^+]_2\,[Cl^-]_2.$$

The concentration of the cation Na^+ in (1) must be equal to the sum of anions present $[R^- + Cl^-]$ in order to maintain electrical neutrality, while on the other side the concentration of Na^+ is only that of the anion Cl^-. It follows that in order for the products just mentioned to be equal, the concentration of sodium ions $[Na^+]$ on the left must be greater than on the right and the reverse must be true for the chlorine ions. That is

$$[Na^+]_1 > [Na^+]_2 \text{ and } [Cl^-]_1 < [Cl^-]_2$$

Thus it is possible to account for the existence of different concentrations of diffusible ions on the two sides of a semi-permeable membrane, the essential condition being the existence of a non-diffusible ion on one side of a membrane which is permeable to the other ions. This difference in concentration of diffusible ions leads to a *difference of potential* (electrical pressure, measured in volts) on the two sides of the membrane and may account for certain electrical phenomena of living matter. An experiment given below in which gelatin chloride solution is dialyzed against weak hydrochloric acid shows that at equilibrium there is in the outer solution a greater acidity (hydrogen ion concentration) than was present at the beginning in either solution. Such equilibria may help to explain the production of acid secretions by the kidneys and gastric cells and of alkaline secretions by the pancreatic and other glands. The Donnan equilibrium helps to explain phenomena of absorption also, and the differences in the concentrations of salts in blood plasma and cerebrospinal fluid, or within and without the red cells of the blood. It is not essential that we have an extraneous membrane separating the two solutions. If we prepare a cube of solid gelatin chloride and place this in a solution of hydrochloric acid the same sort of an equilibrium will develop because the gelatin ions are still practically non-diffusible due to their cohesion.

Proteins contain both acid and basic groups. They may therefore combine with either bases or acids and are said to be amphoteric.

[1] This may also be expressed by the equation for the Donnan equilibrium $x^2 = y(y + z)$ where x is the concentration of Na and Cl ions in the outer solution (2), y the concentration of Na and Cl ions of the free NaCl in the colloidal solution (1) and z the concentration of sodium ions in combination with the non-diffusible anion R. x and y may be determined and z then calculated.

Typical acid and basic groups are the carboxyl ($-COOH$) and amino ($-NH_2$) groups. The formula for the protein gelatin in the unionized state may therefore be represented diagrammatically as $G\begin{matrix}NH_2\\ \diagdown\\ \diagup\\ COOH\end{matrix}$. If an excess of an alkali such as sodium hydroxide be added the alkali depresses the ionization of the amino group and the gelatin functions as an acid to form the salt sodium gelatinate. This salt ionizes with the production of negative gelatin and positive sodium ions. If an electric

$$G\begin{matrix}NH_2\\ \diagdown\\ \diagup\\ COOH\end{matrix} + NaOH \longleftrightarrow G\begin{matrix}NH_2\\ \diagdown\\ \diagup\\ COONa\end{matrix} \longleftrightarrow G\begin{matrix}NH_2\\ \diagdown\\ \diagup\\ COO^-\end{matrix} + Na^+$$

current is passed through such a solution the negatively charged gelatin ions will move toward the positive electrode (anode). This phenomenon is spoken of as *cataphoresis*. If to the gelatin we add, instead of alkali, an excess of hydrochloric acid, the ionization of the carboxyl and other acid groups is depressed and the gelatin functions as a base.

$$G\begin{matrix}NH_2\\ \diagdown\\ \diagup\\ COOH\end{matrix} + HCl \longleftrightarrow G\begin{matrix}NH_2HCl\\ \diagdown\\ \diagup\\ COOH\end{matrix} \longleftrightarrow G\begin{matrix}NH_3^+\\ \diagdown\\ \diagup\\ COOH\end{matrix} + Cl^-$$

In this case the gelatin ions are positive and will migrate in an electric current toward the cathode. If we now adjust the reaction of the solution until the gelatin ionizes equally as an acid and as a base, it does not migrate in an electric field but appears practically unionized. The gelatin is then said to be in the *isoelectric* state, and the reaction of the solution is spoken of as the *isoelectric point* for gelatin. At this point the electrical potential of the particles is at a minimum.

The isoelectric points for proteins are the hydrogen ion concentrations at which their solutions: (*a*) do not migrate in an electrical field; (*b*) either are not combined with acids or bases or are combined in equimolar concentrations with both acid and base; (*c*) show the least hydration and swelling; (*d*) show the lowest solubilities and are most readily precipitated; (*e*) show the greatest tendency toward aggregation; (*f*) show the least viscosity; (*g*) show the lowest osmotic pressure.

Membranes can be prepared which will permit the passage of molecules of the solvent but not of the molecules of a substance in solution. One type of semipermeable membrane is made by depositing copper ferrocyanide in the walls of a porous porcelain cup. If such a

cup be filled with a solution of sugar in water and be immersed in water the sugar molecules can not pass through the membrane. The water molecules however permeate readily and the level of the solution in the cup will rise. The pressure that must be exerted upon the solution within the cup to prevent any increase in volume is called the *osmotic pressure* of the solution. It is dependent upon the number of particles of solute in a given volume of solution and upon the temperature. Its magnitude is indicated by the fact that a 10 per cent solution of cane sugar has at 25°C. an osmotic pressure of 7.6 atmospheres.

Equimolecular concentrations of all substances existing in solution in the molecular (undissociated) state exert the same osmotic pressures. By estimating osmotic pressures it is possible to determine the molecular weights of such substances. The molecular weights of proteins may be determined in this manner using collodion membranes.[1] Ions function as individual particles, and electrolytes therefore give abnormally high osmotic pressures.

Colloidal solutions have low osmotic pressures because of the relatively small number of particles present. Their osmotic pressures are nevertheless important in biology. They are greatly influenced by the presence of electrolytes. No explanation of the cause of osmotic pressure is definitely proven. It may be that the water molecules in solutions are prevented from moving as freely as the molecules in pure water because of the attraction of the solute molecules for them.

The resistance of the surface film of a liquid to rupture is called *surface tension*. This phenomenon appears at the surface (interface) of separation of liquids from other liquids or from solids or gases, and is of physiological importance. Molecules in the center of a liquid are attracted about equally in all directions by the other molecules of the liquid and hence can move about as freely in one direction as in another. At the surface however the molecules will generally be attracted more strongly inward than outward, will have less freedom of movement and a layer of greater density is formed which is called the surface film. This film possesses the properties of a solid and is resistant to rupture. The surface tension of water is reduced by soaps, oils, bile salts and by a great variety of other substances, especially those having long carbon chains and few strongly electronegative (−OH, −COOH etc.) or electropositive groups. Substances reducing surface tension tend to accumulate in surface films and are readily adsorbed. Salts such as sodium chloride tend to increase surface tension and to become less concentrated in the surface film. The reduction of surface tension

[1] Sørensen: *Compt. rend. trav. Lab. Carlsberg*, 12, 262, 1917.

by bile salts is an important aid in the emulsification and digestion of fats.

HYDROGEN ION CONCENTRATION

In considering the influence of acidity on colloidal phenomena, such as viscosity, osmotic pressure, the Donnan effect, etc., it is important to recognize that there are two senses to the modern use of the term, one signifying the *quantity* factor (titratable or total acidity) and the other the *intensity* factor (hydrogen ion concentration). Thus while the total acidity (amount of sodium hydroxide required for neutralization) of 25 c.c. of tenth normal acetic acid is the same as that of 25 c.c. of tenth normal hydrochloric acid, the concentration of free hydrogen ions in the latter is about 100 times as great as in the former. It is the concentration of hydrogen ions which determines the difference between strong and weak acids.

A neutral solution is one which contains equal numbers of hydrogen and hydroxyl ions. An acid solution has a preponderance of hydrogen ions and an alkaline solution an excess of hydroxyl ions. Pure water is a neutral solution. It is dissociated to an extremely small extent according to the reaction:

$$H_2O \longleftrightarrow H^+ + OH^-$$

A reversible reaction of the type

$$A + B \longleftrightarrow C + D$$

proceeds from left to right at a velocity (v_1) which is proportional to the concentrations of A and B, *i.e.* $v_1 = k_1 [A] [B]$. Similarly the opposite reaction takes place at a rate (v_2) proportional to the concentrations of C and D, *i.e.* $v_2 = k_2 [C] [D]$. At a state of equilibrium the velocities in opposing directions are equal and hence $k_1 [A] [B] = k_2 [C] [D]$ or $\frac{[C] [D]}{[A] [B]} = \frac{k_1}{k_2} = K$. This is an expression of the *law of mass action* and states that for a reversible reaction the ratio of the product of the concentrations of the reacting substances on one side of the equation to that of the product of the concentrations of the substances on the other side is constant. That is

$$\frac{[C] [D]}{[A] [B]} = K$$

Applying this to the dissociation reaction of water we have $\frac{[H^+] [OH^-]}{[H_2O]} = K$. The concentrations of H^+ and OH^- in pure water or

in *dilute* aqueous solutions are however so small compared with the concentration of *undissociated* water molecules, that the concentration of the latter, $[H_2O]$, may be considered as constant, and the equation then becomes $[H^+] \times [OH^-] = K[H_2O] = K_W$. That is the *product* of the concentrations of the H and OH ions is constant. The value of K_W has been found to be 1×10^{-14}. That is $[H^+] \times [OH^-] = 1 \times 10^{-14}$. In pure water for every hydrogen ion set free an hydroxyl ion must also be liberated so that the concentrations of the two ions remains equal, that is, $[H^+] = [OH^-]$. Therefore $[H^+] \times [OH^-] = [H^+]^2 = [OH^-]^2 = 1 \times 10^{-14}$. Then $[H^+] = [OH^-] = \sqrt{1 \times 10^{-14}} = 1 \times 10^{-7}$. That is pure water or a neutral solution contains approximately 1×10^{-7} moles[1] of H or OH ions per liter and is a 1/10,000,000 N solution of H or OH ions. Hydrogen ion concentrations are now generally expressed, for practical as well as theoretical reasons, as their logarithms with the sign reversed and indicated by the term pH. That is $pH = -\log [H^+]$; or $[H^+] = 10^{-pH}$. Thus the logarithm of 1×10^{-7} is -7.0 and the pH is 7.0. To take another example the hydrogen ion concentration of 0.1 N HCl which is 91 per cent dissociated is 9.1×10^{-2}. The logarithm of any product is equal to the sum of the logarithms of the numbers concerned. In this case then $\log (9.1 \times 10^{-2})$ equals $\log 9.1 + \log 10^{-2}$ or (consulting tables of logarithms[2]) equals $0.96 + (-2) = -1.04$. The pH, being the negative log of the hydrogen ion concentration, equals $-(-1.04)$ or 1.04. To convert pH values to hydrogen ion concentrations the procedure is reversed, *e.g.* in the case of pH 1.04, the $[H^+] = 10^{-1.04} = 10^{-2 + 0.96} = 10^{-2} \times 10^{0.96}$. The logarithm of a number is the power to which 10 must be raised to give the number, hence the value of $10^{0.96}$ is obtained by looking up the number whose logarithm is 0.96 (*i.e.*, antilog 0.96) which is found to be 9.1. pH 1.04 is therefore equal to $[H^+]$ of 9.1×10^{-2}.

As the product of the H and OH ion concentrations is constant at 1×10^{-14} when the $[H^+]$ increases from 1×10^{-7} [pH 7.0] to 1×10^{-4} [pH 4.0] the $[OH^-]$ decreases to 1×10^{-10} [pOH 10]. The sum pH + pOH always equals 14. According to this nomenclature an increase in pH indicates a decrease in hydrogen ion concentration or true acidity.

Since the hydrogen ion concentration varies in a definite reciprocal manner with hydroxyl ion concentration, the pH scale may be used to express degree of alkalinity as well as acidity.

[1] A mole (or gram ion) of hydrogen is 1 gram; of hydroxyl, 16 + 1 = 17 grams.
[2] For tables of logarithms see Appendix.

Normality of $[H^+]$	pH		pOH	Normality of $[OH^-]$
N/10	1	Acidity	13	
N/1,000	3		11	
N/1,000,000	6		8	
N/10,000,000	7	Neutrality	7	N/10,000,000
	8	Alkalinity	6	N/1,000,000
	11		3	N/1,000
	13		1	N/10

Hydrogen Ion Concentration and Titratable Acidity.—Indicators are substances which undergo changes in color with alterations of the reaction of solutions containing them. They are either weak bases or weak acids and possess different colors in the ionized and unionized forms. Phenol red for example is a weak acid whose molecules have a yellow color, but whose anions have a red color. In acid solutions [pH 6.6 or less] the ionization of the indicator is depressed so that it exists almost entirely in the yellow form. In alkaline solutions [pH 8 or greater] the indicator exists chiefly as a salt which ionizes freely giving a red color to the solution. At intermediate reactions the color is some shade of orange depending upon the relative proportions of the yellow and red forms present. Different indicators change color at different values of pH because like other weak acids and bases they have different dissociation constants (see p. 19). The change in color of an indicator when it ionizes is believed to be associated with a tautomeric change in its molecular structure.

The acidity or alkalinity of a solution may be determined in two different ways by means of indicators. One method is by titration with standard alkali or acid using the indicator to determine the end point of the titration. For this purpose the indicator should be one which gives a sharp color change when an equivalent amount of the standard acid or alkali has been added. At this point the reaction of the solution may be practically neutral (pH7) so that an indicator changing color at about this reaction (litmus or rosolic acid) would be suitable to use. On the other hand if we add 20 c.c. of 0.1 N NaOH to 20 c.c. of 0.1 N acetic acid the solution will not be neutral but slightly alkaline due to *hydrolysis*. The sodium acetate formed will react with the water to produce some NaOH and an equivalent amount of acetic acid. Acetic acid is a weak acid and will dissociate few hydrogen ions while NaOH is a strong base and dissociates to a very considerable extent with the production of OH ions which will thus be in excess and the

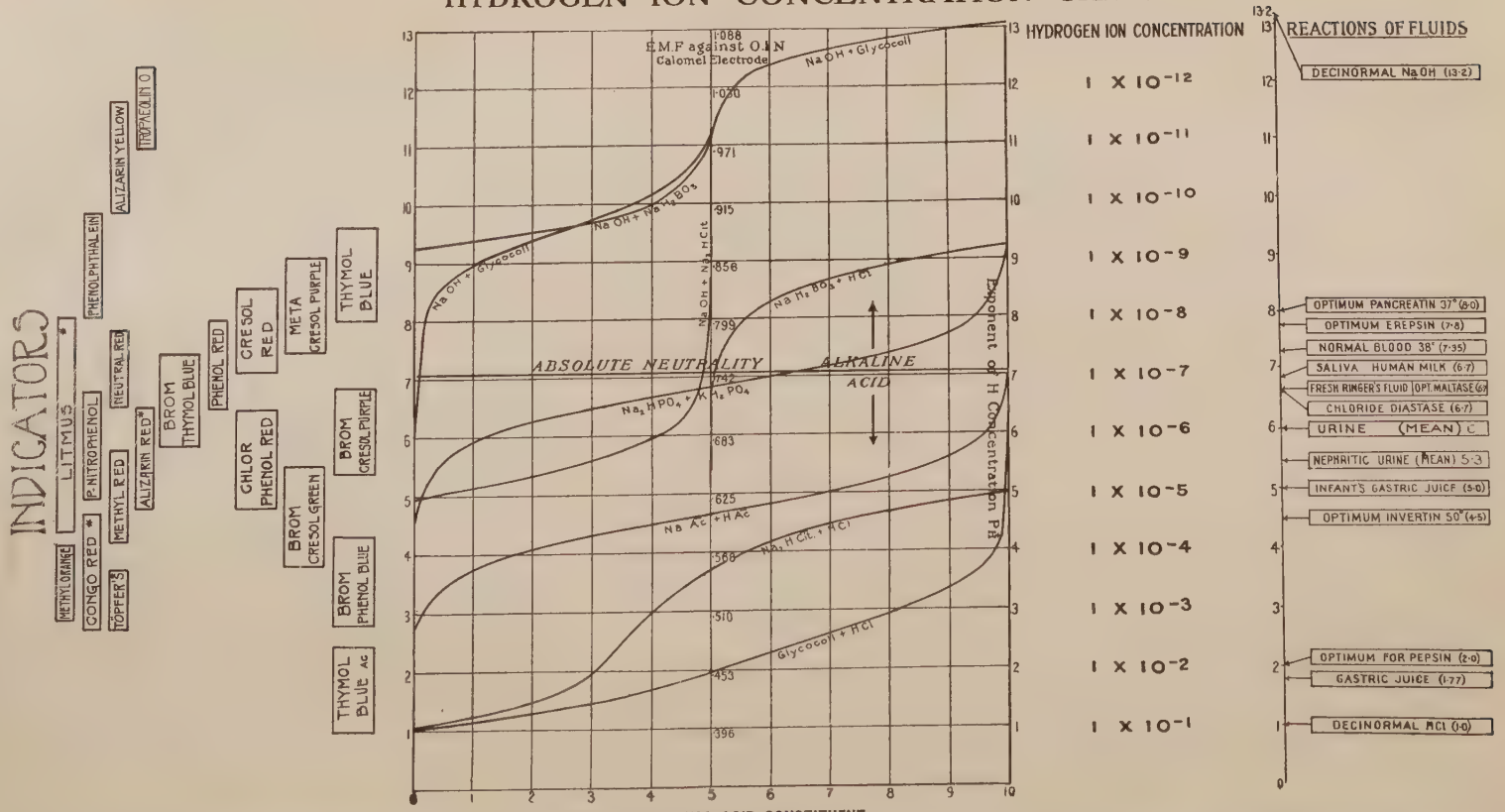
solution will be alkaline. In titrating weak acids such as acetic and most other organic acids it is therefore necessary to use an indicator which changes color in a slightly alkaline reaction. Phenolphthalein has been found to be suitable. In the same way for the titration of weak bases such as ammonia an indicator changing color in acid reaction is required. Congo red and methyl orange are often used in such cases. If a strong base such as NaOH is titrated with a strong acid such as HCl almost any common indicator may be used, as one drop of 0.1 N solutions of these will throw the hydrogen ion concentration so far beyond that of neutrality as to pass the turning point of any of these indicators. For further discussion see electrometric titration method p. 32.

Titration does not, however, enable us to determine in most cases the true acidity of a solution, that is, its hydrogen ion concentration. In the case of strong acids and bases fairly accurate results for the true acidity may be obtained in this way. In the case of weak acids or bases the titration values may give but slight information as to the true acidity. Thus in the case of a slightly dissociated acid, such as acetic acid, as fast as the acidity due to its dissociated hydrogen ions is neutralized the undissociated acid ionizes further and the titration value finally obtained represents the total acid present at the beginning both ionized ond unionized. Salts of strong acids and very weak bases and *vice versa* also hydrolyze during the course of the titration and the values brained in no sense represent the true acidity.

Hydrogen ion concentrations may be determined through a certain range by means of indicators. The unknown solution is treated with a few drops of indicator and the color obtained compared with that produced with the same amount of indicator and a solution of known hydrogen ion concentration. If the same tint is produced in both cases the two acidities are the same. This is of course only true when the indicator chosen is a suitable one, that is, one that shows definite color changes in hydrogen ion concentrations in the neighborhood of that of the unknown. The choice of indicators for this purpose is somewhat different than that for titration purposes. For use in the determination of the hydrogen ion concentration of a solution we need an indicator showing a very gradual change in color through a given range, one which is not readily affected by the presence of neutral salts or other substances likely to be present, and the color of which does not fade too rapidly. The ranges through which a number of indicators may be used with satisfactory results for the determination of hydrogen ion concentrations is indicated in the chart (Fig. 1). The sulphonephthalein series of Clark and Lubs are especially brilliant and reliable.

FIG. 1.

HYDROGEN ION CONCENTRATION CHART



This chart is in sllghtly modified form, one prepared by Walpole from a large amount of data accumulated by Sørensen, Walpole himself, Michaelis, and a number of other authors. See Walpole, *Biochem. J.*, 8, 628, 1914. Indicators marked * are used in titrations but not for pH determinations.

The chart also indicates how standard solutions of definite hydrogen ion concentrations may be made up from a series of stock solutions, by mixing in various proportions. The stock solutions indicated on the chart were suggested by Sørensen and are as follows: 0.10 N HCl; 0.10 N NaOH: 7.505 gm. glycocoll plus 5.85 gm. NaCl per liter: 11.876 gm. $Na_2HPO_4.2H_2O$ per liter:[1] 9.078 gm. KH_2PO_4 per liter: 21.008 gm. citric acid in 1 liter of 0.20 N NaOH: 12.404 gm. boric acid in 1 liter of 0.10 N NaOH. The other solutions are 0.20 N sodium acetate and 0.20 N acetic acid. Solutions of known hydrogen ion concentration are prepared from these by mixing in the proportions indicated on the chart, the abscissæ representing parts of the more alkaline or less acid constituent. Thus a mixture of seven volumes of the sodium acetate stock solution with three volumes of the stock acetic acid solution gives a mixture with a hydrogen ion concentration of 1×10^{-5} (pH 5.0). The mixtures are most satisfactory through the ranges where the hydrogen ion concentration changes most gradually, that is, through the flatter portions of the curves. The phosphate mixtures covering the range 5.3–8.0 are especially useful in biological work. The standard buffer solutions of Clark and Lubs may also be used.

Buffer Solutions of Clark and Lubs[2]

Group 1.—To 50 c.c. of M/5 KCl add the indicated number of c.c. of M/5 HCl and dilute to 200 c.c. Indicator: thymol blue.

pH	HCl	pH	HCl	pH	HCl	pH	HCl
1.2	64.5	1.6	26.3	2.0	10.6	2.2	6.7
1.4	41.5	1.8	16.6				

Group 2.—To 50 c.c. of 0.2 M acid potassium phthalate add the indicated number of c.c. of 0.2 N HCl and dilute to 200 c.c. Indicators: thymol blue and brom-phenol blue.

[1] This sodium phosphate is prepared by drying the powdered crystals containing 12 molecules of water by exposure to air at room temperature in shallow dishes for two weeks or more. To further insure dryness portions of the salt may then be placed in an incubator at 36–38°C. for one or two days.

The water used in preparing these solutions should be distilled water that has been boiled in a Pyrex flask to get rid of CO_2. Stopper the flask while still hot with a rubber stopper carrying a soda lime tube and allow to cool. The sodium hydroxide must be carbonate free (see Clark and Lubs solutions). Protect solutions from the CO_2 of the air.

[2] The constituent solutions are prepared as follows:

0.2 M *potassium chloride.* Dissolve 14.912 gm. in distilled water and make up to 1 liter. The salt should be recrystallized and dried at about 120°C. for two days.

0.2 M *acid potassium phthalate.* Dissolve 40.828 gm. in distilled water and make up to 1 liter. The salt should be recrystallized from distilled water and dried at 110°–115° C. for some hours.

0.2 M *acid potassium phosphate.* Dissolve 27.232 gm. in distilled water and make up

pH	HCl	pH	HCl	pH	HCl	pH	HCl
2.2	46.70	2.8	26.42	3.2	14.70	3.6	5.97
2.4	39.60	3.0	20.32	3.4	9.90	3.8	2.63
2.6	32.95						

Group 3.—To 50 c.c. of 0.2 M acid potassium phthalate add the indicated number of c.c. of 0.2 N NaOH and dilute to 200 c.c. Indicators: brom-phenol blue, brom-cresol green and brom-cresol purple.

pH	NaOH	pH	NaOH	pH	NaOH	pH	NaOH
4.0	0.40	4.6	12.15	5.2	29.95	5.8	43.00
4.2	3.70	4.8	17.70	5.4	35.45	6.0	45.45
4.4	7.50	5.0	23.85	5.6	39.85	6.2	47.00

Group 4.—To 50 c.c. of 0.2 M acid potassium phosphate add the indicated number of c.c. of 0.2 N NaOH and dilute to 200 c.c. Indicators: brom-cresol purple, brom-thymol blue, and phenol red.

pH	NaOH	pH	NaOH	pH	NaOH	pH	NaOH
5.8	3.72	6.4	12.60	7.0	29.63	7.6	42.80
6.0	5.70	6.6	17.80	7.2	35.00	7.8	45.20
6.2	8.60	6.8	23.65	7.4	39.50	8.0	46.80

to 1 liter. The salt should be recrystallized from distilled water and dried at 110°–115° C. for some hours.

0.2 M *boric acid in* 0.2 M *potassium chloride*. Dissolve 12.4048 gm. of air dried boric acid and 14.912 gm. pure KCl in distilled water and make up to 1 liter.

0.2 N *sodium hydroxide*. Dissolve 100 grams of the best NaOH in 100 c.c. of distilled water in an Erlenmeyer flask (Pyrex). Cover the mouth of the flask with tin foil, and allow the solution to stand overnight till the carbonate has settled. Cut a hardened filter paper to fit a Buchner funnel. Treat it with warm, strong (1 : 1) NaOH solution. Decant the soda and wash the paper first with absolute alcohol, then with dilute alcohol and finally with large quantities of distilled water. Place the paper on the Buchner funnel and apply gentle suction until the greater part of the water has evaporated. Now pour the concentrated alkali upon the middle of the paper, spread it with a glass rod, and filter under suction. The clear solution is now diluted quickly with cold distilled water, that has recently been boiled to remove CO_2, to make approximately N NaOH (about 50 c.c. per liter). Ten c.c. of this is withdrawn and roughly standardized against N HCl. It is then diluted till it is approximately 0.2 N with CO_2–free water and the solution poured into a paraffined bottle, to which a burette and soda-lime guard tubes have been attached. The solution is then accurately standardized against weighed amounts of the pure acid potassium phthalate. To do this accurately weigh up about 1.6 gm. of the salt, dissolve in about 30 c.c. of distilled water, add phenolphthalein and titrate with the alkali till a faint but distinct and permanent pink is developed. A current of CO_2 free air should be blown through the solution during the titration.

$$\frac{\text{Grams of phthalate used} \times 1000}{204.14 \times \text{c.c. NaOH required}} = \text{Normality of NaOH.}$$

It is not necessary to make exactly 0.2 N, but the true strength must be considered in making standards.

0.2 N *hydrochloric acid.*—Dilute concentrated HCl to 20 per cent. Distill, dilute distillate, and standardize against the standard soda, using methyl red as the indicator. Clark: Determination of Hydrogen Ions, Baltimore, 1925.

Group 5.—To 50 c.c. of 0.2 M boric acid in 0.2 M KCl add the indicated number of c.c. of 0.2 N NaOH and dilute to 200 c.c. Indicators: cresol red and thymol blue.

pH	NaOH	pH	NaOH	pH	NaOH	pH	NaOH
7.8	2.61	8.4	8.50	9.0	21.30	9.6	36.85
8.0	3.97	8.6	12.00	9.2	26.70	9.8	40.80
8.2	5.90	8.8	16.30	9.4	32.00	10.0	43.90

For experiments on the determination of hydrogen ion concentration see p. 24 *et seq.*

EXPERIMENTS

A. METHODS FOR THE PREPARATION OF COLLOIDAL SOLUTIONS

1. Preparation of Colloidal Solutions of Prussian Blue and of Arsenious Sulphide by Double Decomposition.—To 10 c.c. of N/50 potassium ferrocyanide in a beaker add with mixing 10 c.c. of N/50 ferric chloride. Dilute a portion of the mixture and note that there is no precipitate.

Mix 50 c.c. of one per cent arsenious oxide solution (made by boiling the oxide with water) with 50 c.c. of saturated solution of hydrogen sulphide. If turbid, filter through a folded filter. This is a negative colloid.

2. Preparation of Colloidal Ferric Hydroxide by Hydrolysis.—To 200 c.c. of boiling water add one c.c. of 33 per cent ferric chloride solution. Note the beautiful reddish brown color. This is a positively charged colloid.

3. Preparation of a Gold Sol by Reduction.—To 100 c.c. of pure redistilled water add 1 c.c. of one per cent gold chloride [$HAuCl_4 \cdot 3H_2O$] solution and 5 c.c. of 0.5 per cent potassium carbonate solution. Heat to boiling, remove from flame and add two drops of 20 per cent formaldehyde solution. If necessary heat again and add one more drop. Observe the changes in color as the particles of metallic gold become larger and larger.

4. Preparation of Colloidal Platinum by the Electrical Method.—Connect two short platinum wires (1–3 mm. cross section) with stout copper wires and pass through pieces of glass tubing to serve as handles. Connect both wires with terminals of 110 volt lighting circuit with a lamp bank of five 100 watt lamps in parallel (to cut down current to about 5 amperes). Place pure distilled water in a crystallizing dish about four inches in diameter, add a very slight trace of hydrochloric acid and immerse the two platinum tips. Bring the tips together and then separate by about 1–2 mm. An arc develops. Maintain this for several minutes. Filter.

5. Preparation of Emulsoid Solutions.—Prepare solutions of gelatin (5 per cent), agar (1 per cent), starch (2 per cent) and soap (2 per cent) with the aid of heat. On cooling jellies are produced. Heat again and notice that the gels liquefy.

B. PHYSICO-CHEMICAL PROPERTIES OF SOLUTIONS

1. Diffusion through Membranes.—Prepare a collodion bag by pouring 10–20 c.c. of collodion into a clean dry Erlenmeyer flask. Rotate flask to form a complete film over inner surface and invert over a ring stand. After ten minutes loosen edges of film with a knife and run water from faucet between membrane and flask. Preserve bag in water. Pour into the bag colloidal Prussian blue or ferric hydroxide solution and immerse in a beaker containing water. Note whether particles pass through the membrane. Repeat with copper sulphate or ferric chloride solution and test solution in beaker for copper or iron.

2. Diffusion through Gels.—Place 5 c.c. of 5 per cent gelatin in each of two test tubes. Cool until the gelatin solidifies. Pour copper sulphate over the gel in one tube and Prussian blue solution over the other. Let stand. Note degree of diffusion of the blue color in the two cases. The experiment may be repeated with eosin and methylene blue, the former a non-colloidal and the latter a colloidal dye.

3. Mutual Precipitation of Colloids.—(a) Mix equal volumes of solution of the negative colloid arsenious sulphide and the positive colloid ferric hydroxide.

(b) Mix equal volumes of aluminum hydroxide cream (See "Reagents and Solutions," Appendix) with arsenious sulphide and with ferric hydroxide.

Aluminum hydroxide is an amphoteric colloid and precipitates all colloids which have been tested except hemoglobin.

4. Precipitation of Colloids by Salts.—To a solution of Prussian blue, ferric hydroxide, or other suspensoid add a few c.c. of 10 per cent sodium chloride solution, and allow to stand. Treat a solution of an emulsoid such as gelatin or starch in the same way. Which are precipitated by the electrolyte? Add an excess of water to the precipitate. Is the colloid reversible? If the emulsoid is not precipitated saturate the solution with solid magnesium sulphate. To some of the precipitate add an excess of water and boil. Is the emulsoid reversible?

5. Demonstration of a Protective Colloid.—To a mixture of three drops of concentrated nitric acid and 5 c.c. N/20 silver nitrate add 5 c.c. N/20 sodium chloride. Note the curdy precipitate. Repeat, but add one c.c. of gelatin solution to the silver nitrate and to the sodium chloride solutions before mixing.

6. Demonstration of Optical Properties of Colloids.—Pass a ray of brilliant light through solutions of typical colloids in beakers. Colloidal solutions of gold, mastic, ferric hydroxide, or arsenious sulphide give good results. Note the turbidities where the light strikes the solutions and compare with pure water and ferric chloride solutions which should contain but traces of dust and other particles. Direct sunlight or an arc lamp may be used. The light should be concentrated by means of a lens so that only a part of the solution is illuminated.

If the cone of light be viewed through a Nicol prism and the prism rotated the field will become alternately light and dark indicating polarization. Solutions of eosin or quinine fluoresce in the light but the light is not polarized as these solutions are not colloidal. Also examine a colloidal gold solution under the ultramicroscope and note the Brownian movement of the particles.

7. The Viscosity (Internal Friction) of Colloidal Solutions.—Fill a ten c.c. pipette with water, suspend it in a vertical position and allow to drain. Count the number of seconds required for emptying. Repeat using a solution of Prussian

blue or ferric hydroxide. Repeat again using a two per cent solution of gelatin. How does the viscosity of the suspensoid compare with that of the emulsoid and with that of pure water? A more accurate form of this apparatus is a viscosimeter of the Ostwald type (see following experiment). A solution of gelatin shows its lowest viscosity (most rapid emptying of the viscosimeter) at its isoelectric point (about pH 4.7).

8. Estimation of Viscosity with Ostwald Viscosimeter.—The apparatus illustrated in Fig. 2 is used and is kept in a constant temperature water bath during the determination. Test with water first. By means of a pipette introduce a suitable exact amount (usually 5 c.c.) of water into bulb *e*. Blow at *f* and force liquid up to mark *c*. The fluid column should still reach to bulb *e*. Allow to flow back and note time with a stop watch for the meniscus to pass from *c* to *d*. Repeat until constant values are obtained. Repeat with gelatin or other colloidal solution. If water requires 60 seconds and gelatin solution 120 seconds the relative viscosity of the gelatin is 2.0.

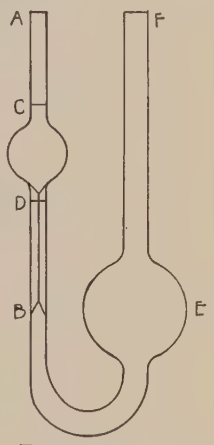


FIG. 2.—OSTWALD VISCOSIMETER.

9. Adsorption by Charcoal.—Caramelize a small amount of sugar by heating in a crucible. Dissolve in 100 c.c. of water, add 5 gm. of animal charcoal, and boil for five minutes. Filter. The filtrate should be colorless. Charcoal is used for clarification in the commercial refining of sugar and in many similar processes. Repeat the experiment using a dilute solution of crystal violet, and extract some of the moist charcoal with alcohol or acetone. Does the dye enter into a firm combination with the charcoal? Acetone and alcohol are more strongly adsorbed than crystal violet and tend to displace it from the charcoal.

10. Adsorption of Dyes by Cellulose.—Filter paper consists of cellulose which in water possesses a negative charge. Suspend strips of acid washed quantitative filter paper so that they dip into 1 per cent solutions of eosin (an acid dye forming true solutions); of Congo red (a dye whose ion has a negative charge) and of methylene blue (a colloidal basic dye). Notice that the water rises on the paper more rapidly than the colloidal dyes which are adsorbed; that the paper dipped in the basic dye becomes more deeply stained, while the eosin being in true solution is but slightly adsorbed. The amount and nature of salts in the paper influence the adsorption.

11. Demonstration of Osmotic Pressure of a True Solution (Cane Sugar).—Make a collodion bag in a 50 c.c. Erlenmeyer flask or in a wide test tube of about 25 c.c. capacity. Wash the bag in running water. By means of a rubber band attach to a rubber stopper, through which is inserted a glass tube about 30 cm. long and having an inside diameter of about 1 mm. With a pipette fill the bag with a 10 per cent solution of cane sugar, insert the glass tube and support so that stopper is at the level of the water in a large beaker. Push in the glass tube so that the level of the sugar solution at the beginning is a little above that of the water. Observe the rise of the sugar solution. Ultimately the sugar will diffuse through the membrane and the solution level in the tube will fall but meanwhile the existence of an osmotic pressure will be demonstrated. Copper ferrocyanide membranes deposited in porcelain cups are truly semipermeable,

and are necessary for quantitative determination of the osmotic pressure of true solutions.

12. Demonstration of Osmotic Pressure of Colloidal Solutions.—The bag employed in the preceding experiment or a similar one may be used and the determination is made in the same way. The highest point of the column is generally reached in 5 or 6 hours. Collodion is not permeable to proteins so a true measure of their osmotic pressure may be obtained using membranes of this type. The pressures attained however are very much lower. A one per cent solution of gelatin may give a rise of from 25 to 50 mm.

The introduction of 1 c.c. of N HCl into the bag for each 50 c.c. of solution and addition of an equivalent amount to the water outside will lead to much higher values for osmotic pressure (see experiment on Donnan equilibrium below and discussion p. 4). The presence of diffusible ions greatly influences the osmotic pressure of protein solutions.

13. Demonstration of Donnan Equilibrium using a Collodion Membrane.—Introduce 100 c.c. of 2 per cent gelatin solution into one 200 c.c. flask and 100 c.c. of distilled water into another. Add one c.c. of 0.5 per cent solution of bromphenol blue to each. Add 0.02 per cent hydrochloric acid to the water drop by drop until the blue color changes to a reddish purple (pH about 3.8). Add 0.2 per cent hydrochloric acid to the gelatin solution until a similar shade of color is obtained. The concentration of hydrogen ions in the two solutions is now the same. Make a collodion bag of 250 c.c. capacity. Pour the gelatin solution into this and suspend in the water solution contained in a 400 c.c. beaker. Let stand. The gelatin solution should gradually turn to a bluish violet indicating a lowered acidity and the outside solution should turn yellow indicating the passage of hydrogen ions into it. The gelatin does not diffuse. The experiment shows how the presence of non-diffusible ions (gelatin) may bring about an unequal distribution of diffusible ions (H^+ and Cl^-) on the two sides of a membrane.

If the mixtures are allowed to stand for some time dye may be adsorbed by the membrane. It is best to pour some of each solution into similar test tubes and add one more drop of indicator to each before comparing.

That the solution within the bag exerts a considerable osmotic pressure can be shown by connecting the bag with a rubber stopper containing a long glass tube as in the experiment on the osmotic pressure of colloids, above. That a definite difference of potential exists between the solutions within and without the bag can be shown by connecting the two through calomel electrodes with instruments for measuring electromotive force.[1]

14. Demonstration of Donnan Equilibrium between a Gel and a Solution without the Use of a Collodion Membrane.—Prepare 100 c.c. of a 5 per cent solution of gelatin. Add one c.c. of a 0.5 per cent solution of bromthymol blue. Add 0.5 per cent NaOH drop by drop until a reddish violet color is obtained. Pour into a 250 c.c. beaker and allow to solidify. Over the gel pour 100 c.c. of distilled water containing the same amount of indicator and brought to the same pH. Let stand and note changes in color of gel and of solution. Is the presence of an extraneous membrane essential for the development of the Donnan equilibrium? This experiment may also be tried using cubes of gelatin.

15. Isoelectric Point and Solubility of Casein.[2]—Introduce into a 50 c.c. volumetric flask 0.25 gm. pure casein. Add about 20 c.c. of water and exactly

[1] See p. 30 and Loeb: Proteins and the Theory of Colloidal Behavior, New York, 1924.
[2] Michaelis and Pechstein: *Biochem. Zeitschr.*, 46,270, 1912.

5 c.c. N NaOH. When solution is complete add exactly 5 c.c. N acetic acid and dilute to 50 c.c. Mix well. This is a solution of casein in N sodium acetate. Set up a series of nine tubes as follows:

No.	1	2	3	4	5	6	7	8	9
Distilled water c.c.	8.38	7.75	8.75	8.5	8	7	5	1	7.4
0.01 N acetic acid c.c.	0.62	1.25							
0.1 N acetic acid c.c.			0.25	0.5	1	2	4	8	
1.0 N acetic acid c.c.									1.6

Add to each tube 1 c.c. of the casein-sodium acetate solution, blowing in from a pipette and shaking the tubes immediately. Note the turbidities just after mixing and after 10 and 30 minutes. Record results as below indicating no turbidity by 0, degrees of turbidity by + signs, and degrees of precipitation by ×.

No.	1	2	3	4	5	6	7	8	9
pH	5.9	5.6	5.3	5.0	4.7	4.4	4.1	3.8	3.5
Turbidity	0	0	+	++	+++	++	+	+	0
After 5 minutes	0	0	+	+++	×××	××	++	+	0

The precipitation should be greatest in tube 5 which shows a pH of 4.7, the isoelectric point and point of least solubility of casein. The acidity in each tube may be actually determined by the electrometric method (see p. 27) or may be calculated from the concentrations of sodium acetate and acetic acid.

According to the law of mass action (p. 8) for a reversible reaction such as $A + B \longleftrightarrow C + D$ the ratio of the product of the concentrations of the reacting substances on one side of the equation to that of the product of the concentrations of the substances on the other side is constant. For the reversible dissociation reaction of the weak acid acetic acid, $CH_3COOH \longleftrightarrow CH_3COO^- + H^+$, we have $\frac{[H^+]\,[CH_3COO^-]}{[CH_3COOH]} = K$. K is called the dissociation constant of the acetic acid. If we add to the acetic acid solution some salt of acetic acid with a strong base, such as sodium acetate, which is highly ionized we increase the concentration of acetate ions. According to the above equation this must result in a lowering of the concentration of the hydrogen ions $[H^+]$ which is brought by a decreased dissociation of the acetic acid. If sufficiently diluted, such a mixture will show a practically complete dissociation of the sodium acetate while the acetic acid will be nearly all in the unionized form. That is, CH_3COO^- is practically equal to the concentration of sodium acetate and CH_3COOH is nearly that of the free acetic acid. Substituting in the above equation $\frac{[H^+]\,[\text{Sodium acetate}]}{[\text{Acetic acid}]} = K$ or $[H^+] = K\frac{[\text{Acetic acid}]}{[\text{Sodium acetate}]}$. For acetic acid $K = 1.85 \times 10^{-5}$.

In the solutions under consideration the sodium acetate is not completely dissociated and it is more accurate to correct for this by multiplying the concen-

tration of sodium acetate by the degree of dissociation which, for 0.1 N sodium acetate is 0.79, and for 0.01 N is 0.87. For present purposes however we may disregard this and consider $K = 2 \times 10^{-5}$.

Then in tube No. 5 which contains 1 c.c. of 0.1 N sodium acetate and 1 c.c. 0.1 N acetic acid:

$$[H^+] = 2 \times 10^{-5} \cdot \frac{0.1}{0.1} = 2 \times 10^{-5}$$

To multiply two figures we add their logarithms. Therefore $\log [H^-] = \log 2 + \log 10^{-5} = .30 - 5.0 = -4.7$. $pH = -\log [H^+] = 4.7$.

16. Isoelectric Point and Precipitation of Gelatin by Alcohol.[1]—Gelatin and many other proteins are quite soluble in water even at their isoelectric points. They do however more readily precipitate at this point if some precipitating agent such as alcohol is added. The method is applicable to albumins such as serum albumin obtained by dialysis of blood serum in a collodion bag for several days (using toluol as an antiseptic).

Prepare a series of test tubes as follows:

No.	1	2	3	4	5	6	7	8	9
0.1 N sodium acetate c.c.	2	2	2	2	2	2	2	2	2
0.1 N acetic acid c.c.	0.12	0.25	0.5	1	2	4			
1.0 N acetic acid c.c.							0.8	1.6	3.2
Distilled water c.c.	3.88	3.75	3.5	3	2	0	3.2	2.4	0.8
1% gelatin c.c.	2	2	2	2	2	2	2	2	2

Mix contents of tubes well and add 95 per cent alcohol to tube 5 until a very faint cloudiness is produced (ordinarily about 8 c.c. is required). Add the same amount of alcohol to each of the other tubes. Examine after 30 minutes. The results usually obtained with approximate pH for each tube are as follows:

No.	1	2	3	4	5	6	7	8	9
Turbidity	−	−	−	++	+++	±	−	−	−
pH	6.0	5.6	5.3	5.0	4.7	4.4	4.1	3.8	3.5

The isoelectric point for gelatin occurs at about pH 4.7.

17. Influence of Acid on the Swelling of Gelatin.[2]—Proteins are least hydrated at their isoelectric points. The addition of acid, as HCl, to gelatin causes it to swell, the process being one of increased hydration. Ions have a strong affinity for water and the addition of acid increases the ionization of the gelatin. In the case of HCl the H ion tends to increase swelling and the Cl ion to depress it. In high concentrations the latter effect overcomes the former and decreased swelling results. The same result follows the addition of Cl ion as NaCl which hence tends to decrease swelling. The influence of ions and other substances on the hydration of cell proteins is believed to be of great biological importance. Relatively slight changes in pH greatly influence protein swelling.

[1] Pauli: Kolloidchemie der Eiweisskörper, Leipzig, 1920. English translation by Thorne: London, Churchill, 1922.

[2] Chiari: *Biochem. Zeitschr.* 33, 167, 1911.

Prepare a series of test tubes as follows:

No.	1	2	3	4	5	6	7	8	9	10
1.0 N HCl c.c.	16	8	4							
0.1 N HCl c.c.				20	10	5	2.5	1.25	0.62	0.31
Distilled water c.c.	4	12	16	0	10	15	17.5	18.75	19.38	19.69

Cut strips from a sheet of gelatin at least 1 mm. thick of as nearly the same size as possible (5 cm. × 0.5 cm.). Put a strip in each tube. After 24 hours measure the length of the strips. Results similar to the following may be obtained.

No.	1	2	3	4	5	6	7	8	9	10
Length (cms)	6.4	6.6	6.7	7.4	8.3	8.3	7.6	7.0	6.1	5.3

The greatest degree of swelling will usually be found in tubes containing from 0.25 to 0.5 N HCl corresponding to pH values of 1.3 to 1.6. The reaction within the gelatin particles themselves at the point of maximum swelling is however about 3.2.[1] This difference in reaction within and without the gel is a consequence of the Donnan equilibrium (see p. 4) which must be considered in all such cases involving the relations of protein ions to other ions in solution. Instead of gelatin strips, 1 gm. portions of powdered gelatin (between 30 and 50 mesh) may be placed in 100 c.c. graduated cylinders and the relative degree of swelling measured with suitable solutions.[1] The actual pH of the gel in any case may be determined electrometrically.

18. Influence of Acidity on the Solidification of Gelatin.[2]—The isoelectric point for gelatin (pH 4.7) is the point of minimum viscosity of its solutions and the point at which the gelatin most readily solidifies. This is because at this point the gelatin is least soluble in the more liquid (water) phase and hence imparts less viscosity to it. For the same reason there is a greater tendency for the more solid phase (gelatin) to form the supporting network believed to be the basis of a jelly. Viscosity determinations must of course be made on the liquefied gelatin solution.

Prepare a series of test tubes as follows:

No.	1	2	3	4	5	6	7
0.1 N sodium acetate c.c.	1	1	1	1	1	1	1
0.1 N acetic acid c.c.	0	0.06	0.25	1	4		
1.0 N acetic acid c.c.						1.6	6 4
1.0 N sodium hydroxide c.c.	0.05						
Water c.c.	6.95	6.94	6.75	6	3	5.4	0.6
pH (approx.)	8	5.6	5.0	4.6	4.0	3.4	2.8

[1] Loeb: Proteins and Theory of Colloidal Behavoir, New York, 1924.

[2] Michaelis: Practical Physical and Colloid Chemistry, translated by Parsons, W. Heffer and Sons, Cambridge, 1925. See also Michaelis: The Effects of Ions in Colloidal Systems, Baltimore, 1925.

Observations may be repeated by remelting the jellies. Note also the much greater opacity of the gelatin at the isoelectric point, indicating that most of the gelatin is in the solid phase.

Tube No.	1	2	3	4	5	6	7
Time required for solidification (min.)	19	17	16	14	19	25	very long

19. Demonstration of Surface Tension.—Half fill a large, thoroughly cleaned and washed beaker with fresh distilled water. Sprinkle some small particles of camphor over the surface. The particles will move about rapidly on the surface. Camphor lowers the surface tension of water. The tension of the surface film will be lowered more rapidly on one side of the particle than on another because of more rapid solution of the camphor at some points, and the particle will be pulled in the direction of greater tension. Touch a glass rod to a cake of soap and then to the surface of the water. The surface tension is greatly reduced and the particles instantly stop moving. Rinse beaker thoroughly and repeat but touch the glass rod to the oily skin of the face and then to the water surface. Oil also reduces surface tension. The particles will move away from the rod (point of low tension) and will finally stop moving. It has been calculated that a layer of oil one molecule deep is sufficient to produce this effect.

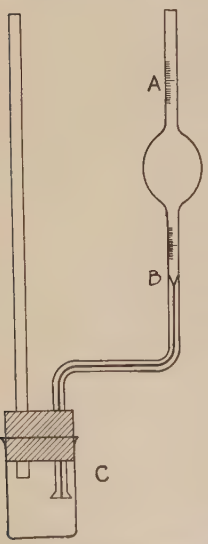


FIG. 3.—STALAGMOMETER.

20. Determination of Relative Surface Tension by the Drop Method.—The surface of a liquid tends to contract so as to produce a minimum area. When flowing through a small opening it therefore tends to form drops which fall away when gravity overcomes the surface tension. The number of drops formed from a given weight or volume of fluid varies therefore with the surface tension.

Procedure.—Set up a Traube stalagmometer as in Fig. 3 so that the whole may be immersed in a constant temperature bath. For approximate comparative results an ordinary 1 c.c. pipette may be clamped in an upright position. Fill the pipette with water above mark A. When the level of the water falls to this mark start counting the number of drops that fall until mark B is reached. By means of the scale divisions above and below A and B (knowing how many divisions correspond to a drop) fractions of a drop may be estimated. If γ represents the relative surface tension of the liquid tested N_w the number of drops of water and N the number of drops of the unknown and S its specific gravity, $\gamma = \frac{N_w \cdot S}{N}$

Compare the surface tensions of water, a very dilute soap solution, and of olive oil, cleaning the instrument thoroughly between tests, and rinsing several times with the solution tested before taking readings. Olive oil has a lower surface tension than water. Soap markedly lowers the surface tension of aqueous solutions.

21. Determination of Surface Tension by the Ring Method of du Noüy.[1] The drop method for determining surface tension described in the preceding experiment is a static method. So also is the measurement of the rise of fluid in a capillary tube. The oscillating jet method is a dynamic method, in that new surfaces are constantly being formed. All methods give practically concordant results in the case of pure liquids, but not in the case of solutions.[2] du Noüy has suggested a static method based on the measurement of the force required to overcome the adherence of a platinum ring to the surface of the liquid in question. The apparatus consists of a platinum ring hanging from a lever arm which can be raised by applying torsion to the wire to which it is fixed. The torsion is measured on a scale which can be calibrated directly in dynes per cm.,

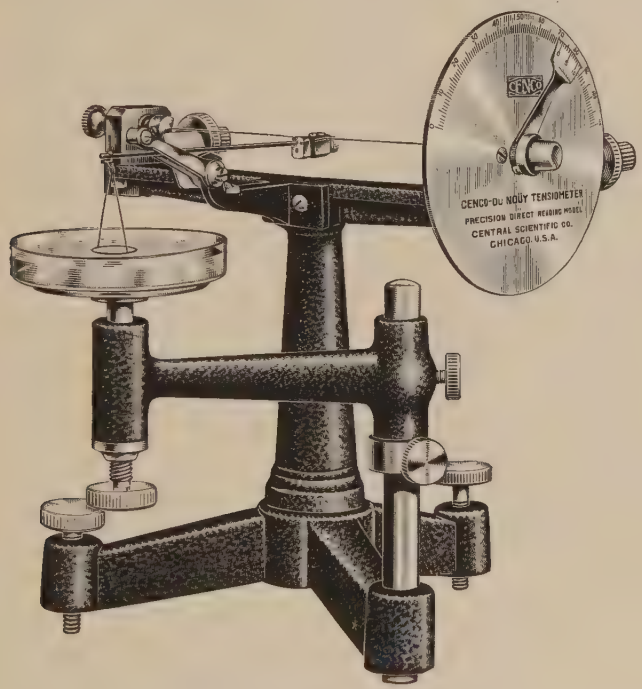


FIG. 4.—DU NOÜY TENSIOMETER.

the absolute unit of surface tension. Determinations of surface tension by this method may be accurately made on 1 c.c. portions of fluid in 20–30 seconds. The method is particularly useful in physiological studies.[3] The surface tension of a resting surface of serum diminishes on standing ("time drop") due to the gradual accumulation of surface active substances (p. 7). This phenomenon has been investigated by du Noüy.[4] Using this apparatus determine the surface tension of the liquids tested in the preceding experiment and compare the results obtained by the two methods (see Fig. 4). This appara-

[1] du Noüy: *J. Gen. Physiol.*, 1,521, 1919.

[2] For a comparative study of different methods, see Ferguson, *Trans. Faraday Soc.*, 17, 370, 1922.

[3] The du Noüy surface tension apparatus with detailed directions for operation may be obtained from houses supplying chemical and physical apparatus.

[4] du Noüy: *Jour. Ed. Med.*, 41, 779, 1925.

tus has been modified to permit the measurement of interfacial tension between two liquids.[1]

22. Colorimetric Determination of Hydrogen Ion Concentration of Unknown Solutions.—Clean thoroughly and rinse with distilled water a number of test tubes of clear (preferably Pyrex) glass of uniform diameter (about 15 mm. inside). Into a series of these tubes introduce 10 c.c. portions of the unknown solution. To one add 10 drops of bromthymol blue solution.[2] Compare the color obtained with the colors given by 10 drops of the indicator and 10 c.c. of 0.5 N HCl (acid color) and with 10 c.c. of 0.1 N NaOH (alkaline color). If a color intermediate between the full acid yellow or alkaline blue is obtained the reaction must lie between pH 6.0 and pH 7.6. Measure out 10 c.c. portions of standard buffer solutions covering this range and add 10 drops of indicator to each. Mix and compare with the unknown in a comparator block (see Fig. 5). Determine which standard has most nearly the same color as the unknown. The pH of this standard will be that of the unknown. If on the other hand the unknown gives the full acid or alkaline color with this first indicator try a second indicator. Thymol blue having the acid range pH 1.2–2.8 and an alkaline range of pH 8.2–9.8 may be used. If the maximum blue or maximum red color with this indicator is obtained (as indicated by testing with HCl and NaOH as before) then the reaction of the unknown is beyond the range of the indicators of this series. Otherwise continue to test indicators until one is found which gives with the unknown solution neither the maximum acid nor maximum alkaline color. Then compare, using this indicator, with standard buffer solutions whose pH lies in the range of the indicator chosen and determine which standard matches the unknown. In measuring out the indicators the dropping pipette should be held perpendicularly so that drops of constant size are obtained. In measuring out buffer solutions

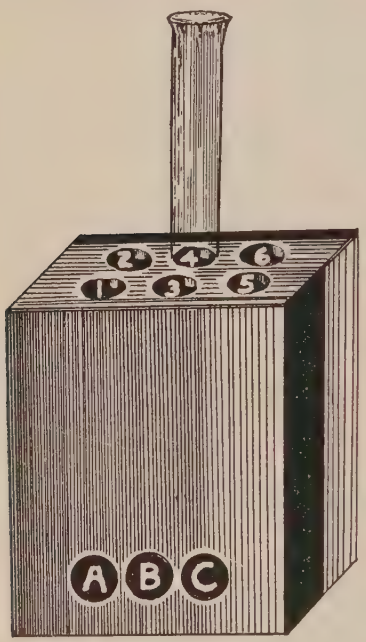


FIG. 5.—COMPARATOR BLOCK.

[1] du Noüy, *J. Gen. Physiol.* 7, 625, 1925.

[2] Indicators may be obtained dry or in prepared solutions from the LaMotte Chemical Products Co., or Hynson, Westcott and Dunning, both of Baltimore, Maryland. 0.04 per cent solutions of all of the Clark-Lubs series of indicators are used. For many purposes 0.04 per cent solutions of the indicators in 95 per cent alcohol may be used. Clark and Lubs prefer to use aqueous solutions of the alkali salts. To prepare these stock solutions rub 0.1 gm. portions of dry powder in an agate mortar with the following quantities of 0.05 N NaOH. Phenol red 5.7 c.c. Brom-phenol blue 3.0 c.c. Cresol red or meta-cresol purple 5.3 c.c. Thymol blue 4.3 c.c. Brom-thymol blue 3.2 c.c. Brom-cresol green 2.9 c.c. Chlor-phenol red 4.7 c.c. If exact equivalents are not desired solution is more readily obtained by using 1.1 equivalents of NaOH. When solution is complete dilute to 25 c.c. with water. Dilute portions of these stock solutions ten times to get 0.04 per cent solutions. Other indicator solutions may be made up as follows: Alizarin yellow R (*p*-nitrobenzene-azo-salicylic acid), methyl orange, tropaeolin o, tropaeolin oo, and tropaeolin ooo in 0.01 per cent aqueous solutions. Methyl red, saturated solution in 50 per cent alcohol. Neutral red, 0.01 per cent in 50 per cent alcohol. *p*-Nitrophenol, 0.04 per cent in 6 per cent alcohol. Phenolphthalein, 0.5 per cent in 50 per cent alcohol. Azolitmin in aqueous solution.

with a pipette the latter should not be blown out and if used for another solution must first be rinsed very thoroughly with distilled water from a flask which has not been blown through (because of the CO_2 in expired air).[1] Color comparisons should be made in a good light using a sheet of white paper as a background.

If the stock solutions of Sørensen are used 10 c.c. portions of standard solutions may be made up from these as required. The stock solutions are measured out from burettes and the proportions required for a certain pH are given in the chart Fig. 1. Let us suppose that the unknown solution has been found to have a pH within the range of phenol red (6.6–8.2). This range is covered by the phosphate buffer mixtures. Measure out 5 c.c. of the acid phosphate and 5 c.c. of the alkaline phosphate, mix, and add 10 drops of indicator. If the same tint is obtained as with the unknown consult the chart. It will be found that this phosphate mixture has a pH of 6.8 and the pH of the unknown must also be 6.8. If the same tint is not obtained on the first attempt make other trials using proportions of 4 : 6, 3 : 7, etc. For other ranges of pH use other indicators and other buffer solutions in the same manner.

If the unknown solution possesses a color of its own either of two procedures may be resorted to. The first method is to add to the standard, before any indicator is added, enough inert coloring matter to match that in the unknown and then add indicator to both.[2] A better method which may also be applied to moderately turbid solutions was suggested by Walpole. The comparator shown in Fig. 5 is used.[3] The colored fluid plus indicator is placed in a test tube in hole "3" and the standard solutions for comparison on each side in holes "1" and "5." Then opposite the standards in holes "2" and "6" are placed tubes containing the colored unknown solution and in hole "4" a tube containing water only. Comparison is made through the apertures "A," "B," and "C."

23. Colorimetric Determination of Hydrogen Ion Concentration Without the Use of Buffer Solutions.[4]—This procedure is most simply carried out using indicators changing from colorless to a single colored form such as phenolphthalein or the nitro-phenols. The depth of color varies with the pH and depends upon the amount of dissociated indicator present. The amount of completely dissociated indicator required to give the same tint as the unknown is a measure of the pH of the latter. The standards are made up by adding measured amounts of indicator solution to portions of solution in which the indicator gives its maximum color (complete dissociation).

[1] It is best to use bottles for the standards the stoppers of which carry 10 c.c. pipettes. Where used by more than one person bulbs may be used to fill the pipettes.

[2] Solutions suitable for this purpose include Bismarck brown, tropaeolin o and tropaeolin oo in 0.02 per cent aqueous solutions, cotton blue 0.01 per cent, methyl violet 0.002 per cent, methyl orange 0.01 per cent all in water, curcumein 0.02 per cent in 60 per cent alcohol and helianthin II 0.01 per cent in 80 per cent alcohol.

[3] A comparator may be made by boring 6 holes in pairs in a block of soft wood the paired holes being as close together as possible and just large enough to hold ordinary test tubes. *Smaller* holes are bored perpendicular to these. Stain black with an alcohol wood stain. A piece of ground glass placed between the light source and the tubes is helpful.

[4] Michaelis and Gyemant: *Biochem. Zeitschr.*, 109, 165, 1920.

To 10 c.c. of unknown solution in a test tube add 1 c.c. of indicator solution.[1] To another tube add 9 c.c. of a solution giving the maximum color with the indicator (e.g. 0.02N NaOH) and then add indicator solution diluted ten times from a micro-burette or Mohr pipette until a color is obtained which on diluting the solution to the same volume as the unknown (11.0 c.c.) matches that of the unknown. If the first standard does not exactly match make a second one.

Calculate the pH from the formula $pH = pK + \log \frac{C}{1 - C}$ where pK is a constant for the indicator (consult table) and C is the ratio of amount of indicator solution (undiluted) added to the unknown to the amount of indicator added to the standard. If 1 c.c. of diluted indicator were used (0.1 c.c. of undiluted) and the indicator is p-nitrophenol (pK 7.18) we have $pH = 7.18 + \log \frac{0.1}{0.9} = 7.18 +$ $(\log 0.1 - \log 0.9) = 7.18 + (-1 - (9.95 - 10)) = 7.18 - 0.95 = 6.23$. A chart giving values of $\log \frac{C}{1 - C}$ is convenient in making calculations.[2]

Other Colorimetric Methods for Determining Hydrogen Ion Concentration. The method without buffers may also be applied to two-color indicators. In this case each standard consists of two tubes set one behind the other in the Walpole comparator. One tube contains for example a known amount of phenol red in alkaline solution (red) and the other in acid solution (yellow). By varying the proportions any shade of this indicator may be matched. For method using this principle see chapter on "Respiratory Metabolism and Neutrality Regulation." The Myers "Bicolorimeter" is convenient for determinations of this type. For an application see chapter on Quantitative Analysis of Urine.

Determinations may be carried out by mixing a drop of unknown or of standard buffer solution with a drop of indicator on an opal glass plate and comparing the colors.[3] Indicator papers may also be used. For application see chapter on "Gastric Analysis."

Comparison of H Ion Concentration and Titratable Acidity.—1. Determine colorimetrically the H ion concentration of an N/100 solution of hydrochloric acid using thymol blue as an indicator and of an N/100 acetic acid using bromcresol green as an indicator. Note the great difference between the true acidities of the two solutions.

[1] Michaelis suggests the following indicators:

Name	Composition	pK 18°C.	Range of pH	Stock solution of Indicator
β-dinitro-phenol. .	1-oxy-2, 6-dinitrobenzol	3.69	2.2–4.0	0.1 gm.: 300 c.c. H_2O
α-dinitro-phenol. .	1-oxy-2, 4-dinitrobenzol	4.06	2.8–4.5	0.1 gm.: 200 c.c. H_2O
γ-dinitro-phenol. .	1-oxy-2, 5-dinitrobenzol	5.15	4.0–5.5	0.1 gm.: 200 c.c. H_2O
p-nitro-phenol. . . .		7.18	5.2–7.0	0.1 gm.: 100 c.c. H_2O
m-nitro-phenol. . . .		8.33	6.7–8.4	0.3 gm.: 100 c.c. H_2O
Phenolphthalein. . .		9.73	8.5–10.5	0.04 gm.: 30 c.c. alcohol + 70 c.c. H_2O

[2] For chart see Michaelis: Practical Physical and Colloid Chemistry. Translated by Parsons. Cambridge, 1925: and Clark: Determination of Hydrogen Ions, Baltimore, 1925.

[3] Felton: *Jour. Biol. Chem.*, 46, 2, 1921.

Titrate 10 c.c. portions of N/100 hydrochloric acid and of N/100 acetic acid with N/100 KOH using phenolphthalein as an indicator. Note that identical results are obtained for the titratable acidities of the two.

2. Determine colorimetrically the H ion concentration of an N/100 KOH solution using thymol blue as an indicator, and of an N/100 ammonia solution using phenolphthalein as an indicator. Note the results and then titrate 10 c.c. portions of both solutions with N/100 HCl using alizarin .

3. Mix equal portions of M/15 potassium dihydrogen phosphate and M/15 disodium phosphate (see chart). Note that the mixture is practically neutral to litmus. Titrate one 10 c.c. portion of this mixture with N/10 KOH, using phenolphthalein as an indicator. Titrate another portion with N/10 HCl solution, using methyl orange.

4. Mix equal volumes of N/5 sodium acetate solution and N/5 acetic acid. Note that the mixture is acid to litmus. Titrate one 10 c.c. portion with N/10 HCl using tropæolin OO as an indicator. Titrate another portion with N/10 KOH using phenolphthalein.

The Electrometric Determination of Hydrogen Ion Concentration

If solid glucose be placed in water, some of the molecules of the solid, being in a continual state of vibration, will escape into the liquid. As the molecules of glucose in solution accumulate, some of them will collide with the particles of solid and be retained. An equilibrium will finally be established in which there is an exact exchange of molecules between the solid and the solution. At this stage, the solution is saturated with glucose and the solution pressure of the solid is said to be equal to the osmotic pressure of the dissolved substance.

If a strip of metal (electrode) is dipped into water, it also tends to dissolve owing to its solution pressure, P. The metal goes into solution as positive ions leaving its electrons (presumably less firmly bound valence electrons) behind as a negative charge on the electrode. This charge, by attracting the positive ions of metal already in solution builds up an electrical double layer at the interface between electrode and solution which opposes the entrance of any more positive ions into the solution and the process of solution ceases, therefore, when this charge becomes large enough to prevent the further separation of positive ions from the metal. Although equilibrium is established before a measurable quantity of the metal dissolves, since the charge on each ion is comparatively large, an appreciable potential difference is developed between the electrode and the solution, which, as will be seen below, may be easily measured.

When a metal is dipped into a solution of one of its salts, the conditions are somewhat altered because the positive ions of the metal already in solution oppose the separation of more ions from the metal. The point of equilibrium, therefore, will depend upon the relative values of

the two opposing forces: the solution pressure, P, of the metal and the osmotic pressure, p, of its ions in solution. We may, therefore, distinguish between three possibilities (see Fig. 6):

1. $P > p$. In this case the metal will continue to send positive ions into the solution until the accumulated charges oppose further action. The metal thus acquires a negative charge relative to the solution.

2. $P < p$. In this case the positive ions in solution will deposit on the metal, yielding to it their positive charges, until the accumulated charges oppose further deposition. The metal thus acquires a positive charge relative to the solution.

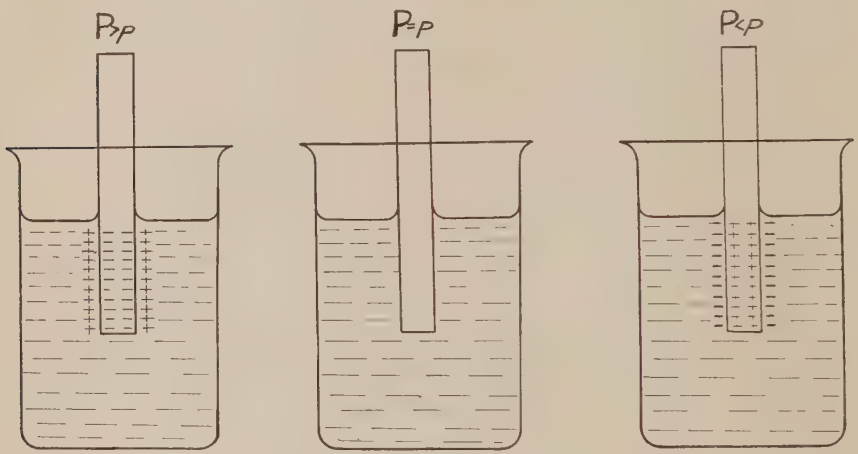


Fig. 6.—Diagram Showing Relations Existing at the Surface of a Metal Dipping into Solutions Containing Its Ions in Different Concentrations.
P = solution pressure of the metal. p = osmotic pressure of metallic ions.

3. $P = p$. In this case there will be neither solution nor deposition and no potential difference will develop between the metal and the solution.

Nernst has shown by thermodynamic reasoning that the potential difference between a metal and a solution of one of its salts, is given by

$$E = \frac{RT}{nF} \ln \frac{P}{p}$$

where E = electrode potential, R = "gas constant" = 8.316 Joules per degree, T = absolute temperature, n = valency of metal ion, F = Faraday = 96,500 coulombs, P = solution pressure and p = osmotic pressure of metal ions in solution. ("ln" is the symbol for the natural logarithm.) From this equation, it becomes evident that, since RT/F is a constant for any given temperature, the magnitude and sign of the electrode potential, E, is determined by n and P, which depend

solely upon the nature of the metal used, and p, which is proportional to the concentration of metal ions in solution. If, therefore, we have two electrodes of the same metal dipping into solutions of different concentrations, the two electrode potentials will be different. And, if we make suitable connections[1] between the two electrodes and the two solutions, an electric current will pass from one electrode to the other, the electromotive force of which will be equal to the difference between the two electrode potentials, *i.e.*

$$\begin{aligned} E &= E_1 - E_2 \\ &= \frac{RT}{nF} \ln \frac{P_1}{p_1} - \frac{RT}{nF} \ln \frac{P_2}{p_2} \\ &= \frac{RT}{nF} (\ln P_1 - \ln p_1 - \ln P_2 + \ln p_2) \\ &= \frac{RT}{nF} \ln \frac{p_2}{p_1} \text{ (since } P_1 = P_2 \text{ for the same metal.)} \end{aligned}$$

But since osmotic pressure is proportional to concentration, we may replace p_1 and p_2 by c_1 and c_2, and get

$$E = \frac{RT}{nF} \ln \frac{c_2}{c_1}$$

An arrangement such as here described is known as a concentration cell, and the above equation permits us to calculate the concentration of metal ions in an unknown solution from the electromotive force developed when that solution is combined in a cell with a similar solution of known ionic concentration.

A strip of platinum, coated with platinum black and saturated with hydrogen gas, acts exactly as the metal electrodes described above when dipped into a solution containing hydrogen ions. We can, therefore, construct a concentration cell by using two such electrodes dipping into two solutions of different hydrogen ion concentrations and, if the concentration of hydrogen ions in one solution is kept at a known, constant value, we can use the electromotive force developed by such a cell to calculate the hydrogen ion concentration in the second, unknown solution. If the concentration of hydrogen ions in the known solution is normal, the equation for the electromotive force becomes $E = \frac{RT}{nF} \ln \frac{1}{[H^+]}$ and substituting the values for R, T, n and F at 18°C. and multiplying by 2.303 to change from the natural to the common system of logarithms,

[1] The two solutions are usually connected by a salt bridge consisting of a U-tube filled with saturated KCl solution. This tends to eliminate any differences of potential which may be built up at the junction of the two solutions by the unequal diffusion of the ions. The ends of the U-tube are plugged with cotton to prevent siphoning of the solution from one vessel to the other. The electrodes are connected by wire in the ordinary manner.

we get $E = 0.058 \log \frac{1}{[H^+]}$ or $E = 0.058 (-\log [H^+])$. Hydrogen ion concentrations, following the convention introduced by Sørensen, are usually expressed in terms of pH where, by definition, $pH = -\log [H^+]$. Therefore, $E = 0.058\, pH$ or $pH = \frac{E}{0.058}$.

In practice it is convenient to use instead of a hydrogen electrode in a normal solution of hydrogen ions an electrode of mercury and calomel which has been standardized against such a solution. The normal hydrogen electrode is 0.25 volts more positive than the saturated calomel electrode so that the difference of potential between the calomel electrode and the unknown will be 0.25 volts greater than that between the latter and the normal hydrogen electrode. Therefore, using the saturated calomel electrode at a temperature of 18°C., $pH = \frac{E - 0.25}{0.058}$. We may read E directly in volts on a voltmeter so that the calculations become very simple. Also, variations of pH with E may be plotted in the form of a curve or the voltmeter may be graduated to read pH directly so that even this calculation is avoided.

A simplified electrometric method for the measurement of hydrogen ion concentration has been described by Meeker and Oser.[1] This method is based on balancing the potential of the unknown solution in a hydrogen electrode half-cell by titrating buffer solutions into a similar opposing half cell. The proportion of the buffers at equilibrium is a measure of the pH in the unknown cell. (See experiment page 34.)

Determination of Hydrogen Ion Concentration by Hildebrand's Method.[2]—Set up the apparatus as indicated in the diagram (Fig. 7). The battery may be an ordinary dry cell. The voltmeter V should have a range of 1.2 volt and scale divisions to 0.01 volt. The portable galvanometer G should have a sensitivity of at least one megohm. A capillary electrometer may be used in place of the galvanometer. The rheostat R may be a tubular wire rheostat having at least 150 turns of wire. K is a contact key for opening and closing the circuit. C is the calomel electrode.[3] H is a hydrogen electrode of the Hildebrand type especially suitable for solutions not containing protein. The Clark electrode is

[1] Meeker and Oser, *Jour. Biol. Chem.* 67, 307, 1926.

[2] Hildebrand: *J. Am. Chem. Soc.* 35, 847, 1913.

[3] Calomel Electrode. Place about 3 c.c. of carefully purified mercury in the bottom of the electrode vessel. Rub together mercury and mercurous chloride (calomel) with a little saturated KCl solution to form a paste. Place a layer of this paste about a half inch thick over the mercury and fill the vessel to the end of the side tube with saturated KCl solution. If it is desired to make a normal or tenth normal calomel electrode corresponding solutions of KCl should be used instead of the saturated solution. It is convenient to make a permanent connection between the upper side arm and a reservoir bottle containing KCl solution. By running a little of the solution through the calomel cell before each series of determinations the side arm may be washed free from materials that may have diffused up into it. The stopcock of this electrode should be tight but should not be greased.

a type frequently employed in biochemical work and may be used with a shaking device to assist in obtaining equilibrium rapidly.[1]

The essential part of the hydrogen electrode is the piece of platinum foil which is to dip into the unknown solution. This must be given a spongy coat of platinum black so that it will retain as much hydrogen as possible and must be saturated with hydrogen just before use.[2]

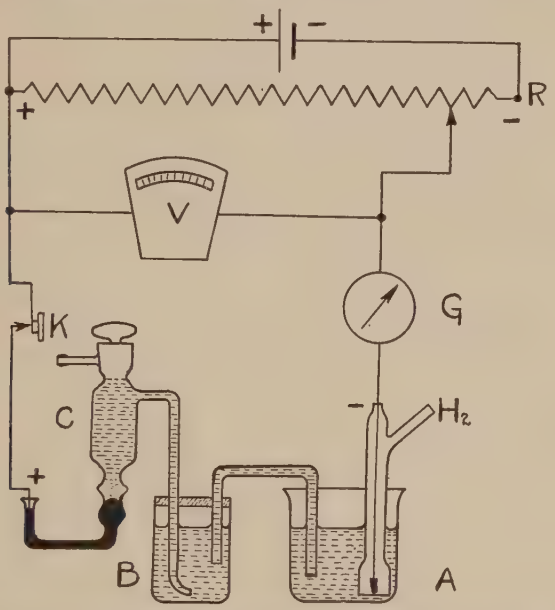


FIG. 7.—SHOWING ARRANGEMENT OF APPARATUS FOR ELECTROMETRIC DETERMINATION OF HYDROGEN ION CONCENTRATION. (*Method of Hildebrand.*)

H is the hydrogen electrode, C the calomel electrode, V a voltmeter, G a galvanometer, R a rheostat, A the beaker containing the unknown solution, and B a connecting vessel.

The unknown solution is placed in beaker A. The calomel electrode is clamped in place as is also the prepared hydrogen electrode. A stream of hydrogen[3] is passed through the side arm of the hydrogen electrode so that it bubbles

[1] McClendon has suggested a special form of electrode for use with blood (see *J. Biol. Chem.*, 24, 519, 1916). De Eds and Hanzlik (*J. Biol, Chem.* 60, 355, 1925) recommend a simple form for use with very small amounts of blood and similar materials. A convenient form of the bubbling type of electrode is that of Bunker (*J. Biol. Chem.* 41, 11, 1920). For method of use of Clark electrode and other details see Clark: Determination of Hydrogen Ions, Baltimore, 1925, also *Jour. Biol. Chem.* 23, 475, 1915.

[2] Clean the platinum electrode thoroughly with chromic acid. Connect with the negative pole of a dry cell and connect another strip of platinum with the positive pole. Dip both in a dilute (two per cent) solution of platinic chloride. Electrolyze for 10–15 minutes frequently reversing the current. Dip into 10 per cent sulphuric acid, with the electrode as the cathode, and let the current run for a few minutes. The platinum is saturated with hydrogen. Wash with distilled water. Keep under distilled water when not in use. The platinum coat should last for several weeks but must be saturated with hydrogen just before use. Instead of a strip of platinum a wire about 1 mm. in diameter slightly flattened at the end may be used. Equilibrium with such an electrode may be brought about more rapidly.

[3] Hydrogen may be purchased in cylinders, or may be prepared by electrolysis of an NaOH solution or from pure zinc. To purify pass through a wash bottle containing alkaline pyrogallol solution and two bottles containing water. Connect one of these bottles with a third tube dipping below the surface of mercury in another vessel. This acts as a safety. With gas tanks a gas regulator for adjusting flow is desirable.

up through the solution at the rate of about two bubbles per second. Beaker B and the connecting tube from A to B contain saturated KCl solution.

Stir the solution well. An automatic stirring device is best for this purpose. Move the sliding contact on the rheostat until the voltage drawn from the dry cell is equal to that produced in unknown solution. This is indicated by the fact that no current will pass through the galvanometer when the contact key K is pressed down and there will be no deflection of the needle. Until a balance is reached the spring contact key should be closed only momentarily by a slight tap. At the balance point read the voltmeter.

Calculation of pH.—See discussion preceding determination. At 18° using saturated calomel electrode $pH = (E - 0.25)/0.058$. If the voltmeter reads for example 0.656 then $pH = (0.656 - 0.25)/0.058 = 7.0$ and the solution possesses a neutral reaction. Corrections for different electrodes and temperatures are given in the following table. This apparatus[1] should have an accuracy of about 0.1 pH. Test the apparatus first using buffer solutions of known pH.[2]

STANDARD VALUES FOR CALOMEL ELECTRODES

(Referred to the normal hydrogen electrode)

Temperature °C.	E_0 for different concentrations of KCl			
	M/10	M/1	Saturated (approximate potential)	$2.303 \frac{RT}{nF}$
18	0.3380	0.2864	0.2506	0.0577
20	0.3379	0.2860	0.2492	0.0581
25	0.3376	0.2848	0.2464	0.0591
30	0.3373	0.2837	0.2437	0.0601
40	0.3360			

Electrometric Titration of Acids and Bases Using Hydrogen Electrode.[3]—In many cases the use of indicators in titration is unsatisfactory because of color or turbidity of solutions to be examined or the presence of buffer substances which make the end point uncertain. The hydrogen electrode may be used in such cases in titrating solutions to a definite acidity or alkalinity. This method also makes it possible

[1] The apparatus described is one of the most easily assembled and is suitable for many purposes. For more accurate work a potentiometer which embodies a bridge, resistance boxes and keys in one apparatus should be used. The potentiometers made by the Leeds and Northrup Co., Philadelphia have been widely used. Assemblies suitable for various purposes with directions for use may be obtained from manufacturers and dealers. See Clark: Determination of Hydrogen Ions, Baltimore, 1925, for more detailed discussion and bibliography.

[2] A suitable solution tenth normal with respect to both acetic acid and sodium acetate may be used. At 18° with the saturated calomel electrode this gives 0.517 v. and with the N/10 electrode 0.6045. Mix 50 c.c. N NaOH and 100 c.c. N acetic acid with water to make 500 c.c.

[3] Kolthoff and Furman: Potentiometric Titrations, John Wiley and Sons, New York, 1926.

to follow the changes in acidity during the course of a titration and gives information as to the character of the acids or bases in solution. Some applications of this method are illustrated in Fig. 8.

Procedure.—The apparatus used is that already described and illustrated in Fig. 7. The unknown solution is placed in a beaker (A), into which are introduced both the hydrogen and calomel electrodes, beaker B and the connecting tube being omitted. Hydrogen is bubbled through the solution and acid

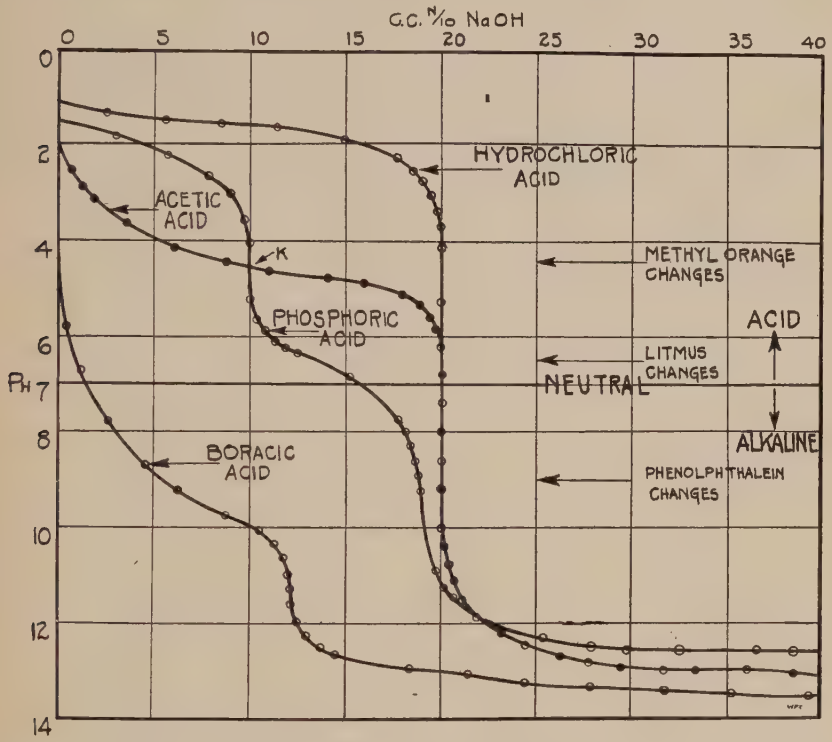


FIG. 8.—TITRATION CURVES OF VARIOUS ACIDS, OBTAINED BY HYDROGEN ELECTRODE MEASUREMENTS.

20 c.c. of 0.1 N hydrochloric and acetic acids used. Phosphoric acid, about ⅔ molar. Boric acid, saturated solution. K is the ionization constant of acetic acid.

or alkali added from a burette with stirring. At intervals read the voltmeter. Plot a curve of voltage or pH against cubic centimeters of acid or alkali added. Neutralization is indicated by a rapid change of voltage or pH. Di- or tribasic acids or bases may show two or more such changes. See curve for titration of phosphoric acid (Fig. 8).

As will be seen from the chart Fig. 8 in the titration of a strong acid such as hydrochloric acid with a strong base such as sodium hydroxide the change in H ion concentration at the end point is very sharp because as long as even 0.1 c.c. of the acid remains unneutralized the pH is still quite low and 0.1 c.c. of 0.1 N NaOH in excess gives a very large increase of pH. Evidently either methyl orange, litmus or

phenolphthalein may be used in the titration. With acetic acid however the beginning $[H^+]$ is lower because this acid is weakly dissociated. The change in pH is at first fairly rapid because the acetate ions of the sodium acetate formed repress the ionization of the acid, but for the same reason the change later becomes slow as the ionization of the acid is almost completely repressed by the larger amounts of acetate formed. When an equivalent amount of alkali has been added the reaction is alkaline due to hydrolysis of the sodium acetate to form acetic acid, nearly all undissociated, and NaOH largely dissociated. The actual end point of the titration is therefore on the alkaline side and phenolphthalein is the proper indicator.

The chart shows also how it is possible to titrate the first hydrogen of phosphoric acid using methyl orange as an indicator and the second using phenolphthalein, while the third cannot be titrated because the change is too gradual. Neither can boric acid be titrated directly.

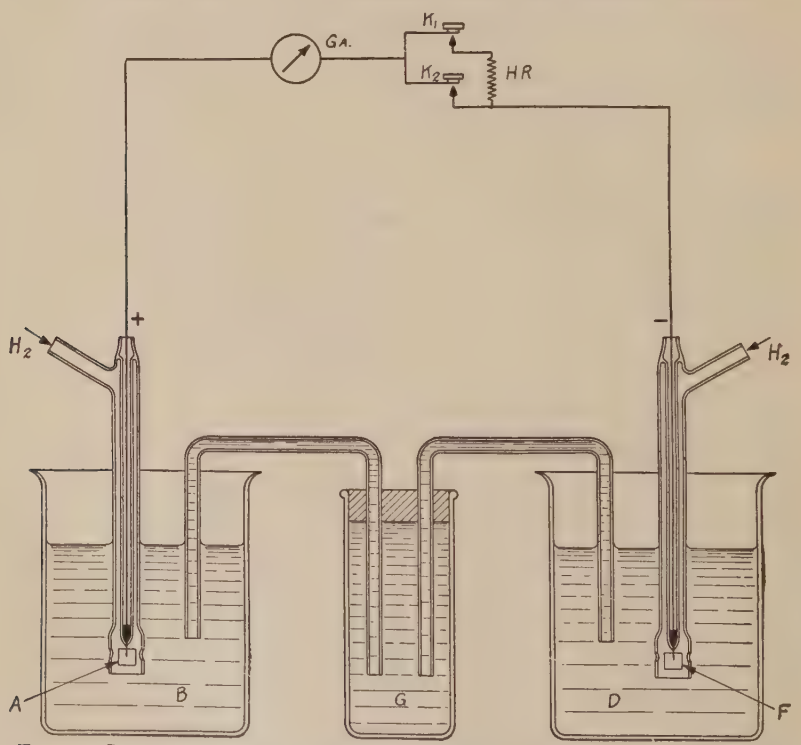


Fig. 9.—Showing Connections for Double Hydrogen Electrode System. A and F are the hydrogen electrodes, B and D the electrode cells, G the salt bridge, Ga the galvanometer, HR a 10,000 ohm resistance coil which is thrown in series with the galvanometer when the tapping key K_1, but not when K_2, is closed.

Determination of H Ion Concentration by Electrometric Titration, Using the Double Hydrogen Electrode System.[1] **Principle.—When the electrical potential**

[1] Meeker and Oser, *Jour. Biol. Chem.*, 67, 307, 1926.

at a hydrogen electrode is balanced by an equal and opposite potential, no current will flow in the circuit. This principle is employed by arranging two hydrogen electrode cells as in Fig. 9, one of which contains the solution whose H ion concentration is to be determined, while into the other is titrated a properly selected buffer system. The proportion of acid and basic constituents in the buffer system which is required to produce a balance, i.e., no deflection of the galvanometer when the circuit is closed, determines the H ion concentration in both cells at the equilibrium point.

Procedure.—Platinize two electrodes as described in footnote p. 31, taking care to have them as nearly alike as possible. A salt bridge may be made in a small U tube by filling with a hot saturated solution of KCl containing about 2 per cent agar. On cooling a gel will form. Arrange the circuit as shown in Fig. 9, using a galvanometer (Leeds and Northrup No. 2320d is suitable)

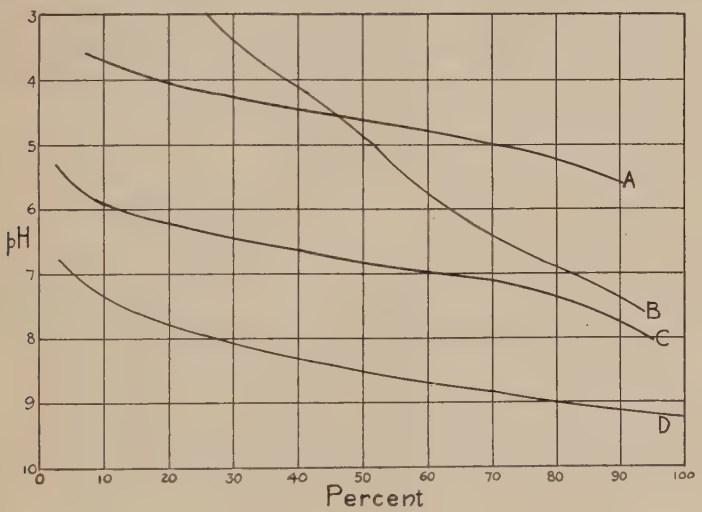


FIG. 10.

A, Walpole's acetate mixtures (per cent sodium acetate).
B, McIlvaine's phosphate-citric acid mixtures (per cent disodium phosphate).
C, Sørensen's phosphate mixtures (per cent disodium phosphate).
D, Palitsch's borax-boric acid mixtures (per cent borax).

and a tapping key (the resistance coil shown in the diagram may be omitted). The solution whose pH is to be measured is introduced into one of the half-cells, and a measured volume (e.g. 10 c.c., of one solution of the buffer pair into the other.[1]

The electrodes are immersed and connected with the supply of hydrogen, which is allowed to bubble through until the electrodes and solutions are completely saturated. The salt bridge is then introduced and the other solution of the buffer pair delivered into the titration cell from a burette. The key is tapped at intervals, more frequently as the galvanometer deflection diminishes.

[1] The selection of the buffer solution depends, on the range of pH in which the unknown solution falls. This can be readily estimated by the use of indicators. For instance, if a solution is "alkaline" to methyl red and "acid" to phenol red, its pH must fall somewhere between the ranges of these two indicators, namely pH 6 to pH 7. In this case buffer solutions are selected covering this range.

At zero deflection the burette reading is taken. The equivalence of the electrodes may be confirmed now by immersing both in the same solution and tapping the key. No current should flow in the circuit.

The pH of the unknown solution, being the same as that of the buffers in the titration cell, at equilibrium, may be obtained from curves constructed for the various buffer systems (Fig. 10) in which the proportion of one of the solutions in the final mixture is plotted against the pH.[1] For example, if 10 c.c. of M/15 KH_2PO_4 are introduced into the titration cell at the beginning and it requires 4.8 c.c. of M/15 Na_2HPO_4 to balance the potential in the unknown cell, then $\frac{4.8}{10 + 4.8} \times 100 = 32.4$ per cent, the proportion of Na_2HPO_4 in the buffer mixture. By referring to the curve for the phosphate mixtures, the pH for this ratio is found to be 6.53. The pH may also be calculated from the equation: $pH = pK_1 + \log \frac{salt}{acid}$, the logarithmic form of the equation for the dissociation constant of a weak acid.[2] (See p. 19.)

Determination of H Ion Concentration by Electrometric Titration, Using the Double Quinhydrone Electrode System.[3] Principle.—The method of balancing

[1] Such curves may be constructed from data given on p. 13 and also in Clark's "Determination of Hydrogen Ions," Baltimore, 1925. See also Chapter XXXI.

[2] This is derived as follows, for the acid HA:

$$K = \frac{H^+ \times A^-}{HA}$$

Therefore

$$H^+ = K \frac{HA}{A^-}$$

In the case of a weak acid and its salt practically all of the A^- originates from the dissociation of the salt, BA. If the degree of dissociation be represented by λ, the concentration of anion $A^- = \lambda BA$.

Hence

$$H^+ = K \frac{HA}{\lambda BA}$$

Since λ varies only slightly over the narrow ranges for which these buffers are used, $\frac{K}{\lambda}$ may be regarded as practically constant, K_1. Therefore

$$H^+ = K_1 \frac{HA}{BA}$$

Taking logarithms,

$$\log H^+ = \log K_1 + \log \frac{HA}{BA}$$

Changing signs,

$$-\log H^+ = -\log K_1 - \log \frac{HA}{BA}$$

$$pH = pK_1 + \log \frac{BA}{HA}$$

pK_1 is obviously the pH at the point where the concentrations of BA and HA are equal (50 per cent dissociation), since $\log 1 = 0$. The pK_1 for the phosphate mixtures is 6.85. Therefore, in the above example,

$$pH = 6.85 + \log \frac{4.8}{10}$$

$$pH = 6.85 + (\log 4.8 - \log 10)$$
$$pH = 6.85 + 0.68 - 1$$
$$pH = 6.53$$

[3] Meeker and Oser, *Jour. Biol. Chem.* 67, 307, 1926

electrical potentials is employed here as in the previous method. The advantage of the quinhydrone electrode is that it dispenses with platinizing electrodes and bubbling hydrogen and therefore makes the method applicable to solutions containing dissolved CO_2. It may be applied to the determination of the pH of urine or blood (see Chapters XVIII and XXXI). Quinhydrone is a rather insoluble crystalline compound prepared from hydroquinone by the action of ferric alum. The quinhydrone in solution dissociates into hydroquinone and quinone, which are in equilibrium according to the equation:

$$\underset{\text{Hydroquinine}}{C_6H_4(OH)_2} \longleftrightarrow \underset{\text{Quinone}}{C_6H_4O_2} + 2H^+ + 2 \text{ electrons}$$

As long as the molecular ratio $C_6H_4(OH)_2$:$C_6H_4O_2$ is 1:1, the potential at a platinum or gold electrode immersed in the solution is dependant only upon the H ion concentration. This condition is not maintained when the solution becomes too alkaline (pH $>$ 8), this being the chief limitation of the quinhydrone electrode method.

Procedure.—Unplatinized platinum or gold electrodes are used. The unknown solution and the measured volume of one solution of the buffer pair are introduced into their respective beakers. An excess (enough to cover the tip of a spatula) of quinhydrone is added to each solution and stirred thoroughly. The salt bridge connection is made, and the second buffer solution titrated from a burette as in the previous method, stirring constantly. The reading of the burette at zero deflection of the galvanometer is noted. The pH is obtained in the manner described above.

Electrometric Oxidation and Reduction Titrations.—When a substance is oxidized it loses electrons, when it is reduced it gains electrons. Thus when Fe^{++} is oxidized to Fe^{+++} there is a loss of one electron or negative charge. The process is therefore similar to that resulting in the loss of a negative electron when hydrogen passes into solution as H^+. If a platinum wire be placed in a solution containing for example ferrous and ferric ions the ferrous ions will tend to give up electrons to the platinum and the ferric ions will tend to take them away. A difference of potential will arise depending upon the concentrations of the two ions. If an oxidizing agent be now added there will be a change of potential, slow at first and then very rapid, as the concentration of ferrous ions becomes very small compared to that of the ferric ions. This rapid change of potential marks the end point of the titration.

Procedure.—The apparatus used is the same as for the electrometric titration of acids and bases. The platinum electrode should however be small and should not be platinized but be kept bright. Keep in 1:1 HCl when not in use. The calomel electrode should be connected to the negative side of the main circuit instead of the positive. Instead of a beaker a flask may be used and a current of CO_2 passed over the surface of the solution (not through it) to prevent oxidation by the air.[1] Run in oxidizing or reducing agent from a burette. Readings of

[1] If desired the voltmeter may be eliminated. In this case adjust rheostat so that there is no galvanometer deflection at the beginning of the titration. The end point is indicated by the galvanometer needle being thrown off the scale.

the voltmeter are taken at intervals and at the end point a drop or two of solution produces a marked change of potential.

Determination of Electrical Conductance of Solutions.—The power of any solution to conduct an electric current depends on the number of ions in the solution and the mobility of those ions. Strong acids and bases or the salts of strong acids and bases are almost completely dissociated in solution in the concentrations commonly met with in physiological fluids. Weak acids and bases and their salts are but slightly dissociated and neutral organic substances other than salts do not ionize.

It follows that the conductance of most biological fluids is dependent upon their content of salts of strong acids and bases, especially sodium and potassium chlorides. This is particularly true because f othe relative abundance of chlorides of sodium and potassium in body fluids. Thus the blood and bile of most mammals are higher in chlorides and possess considerably higher conductances than milk and saliva which are lower in chlorides.

When we study the gastric juice we at once note that while the ash of this fluid is very low as compared with blood, bile, pancreatic juice, etc., its conductance is much higher than that of these fluids. Thus while the specific conductance of bile at 18° is 130×10^{-4} reciprocal ohms, that of blood serum about 110×10^{-4} and that of saliva about 50×10^{-4}, the conductance of pure gastric juice is over 400×10^{-4}. The total chlorine of gastric juice is not notably higher than in blood, bile, or pancreatic juice, but it conducts a current much more readily because of the great mobility of the hydrogen ion. Thus the ionic speed at 18° of the sodium ion is 43.5×10^{-5} cm. per second, of the potassium ion 64.6, of the chlorine ion 65.5 while that of the hydrogen ion is 318. A tenth normal solution of hydrochloric acid at 18° has a specific conductance of 351×10^{-4} as compared with 92.5 for sodium chloride and 111.9 for potassium chloride.

It is easy to understand therefore why the conductivity of gastric contents generally runs parallel with the free hydrochloric acid, although in low acidities, especially following regurgitation of pancreatic juice and bile the alkali chlorides become an important factor. Conductivity determinations are also used for the measurement of the volume of red cells in blood (the plasma conducts, but the red cells do not) and for other studies on blood and urine. Studies on suspensions of other types of cells also throw light on the conductivity of protoplasm and the permeability of the cells for ions.

Procedure.—Set up apparatus as indicated in Fig. 11. The conductivity cell C contains the unknown solution between two platinum electrodes. A cell

suitable for biochemical work and requiring but small amounts of material is illustrated in Fig. 12. R is a resistance box, AB is a resistance wire mounted on a meter stick or similar scale. D is a sliding contact, T is a telephone headset and E a source of alternating current.[1]

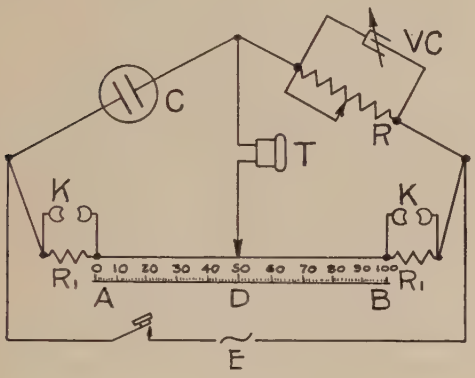


FIG. 11.

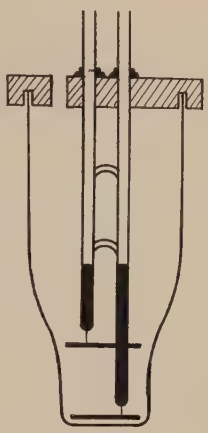


FIG. 12.

FIG. 11.—DIAGRAM OF APPARATUS FOR DETERMINATION OF ELECTRICAL CONDUCTIVITY. C is the conductivity cell, R the resistance box, AB the slide wire resistance, T a telephone, and E a source of alternating current.

FIG. 12.—CONDUCTIVITY CELL.

[1] A suitable assembly may consist of: A *cell* of the type illustrated. For solutions of high resistance the electrodes should be large and close together and for solutions of low resistance they may be small and be placed further apart. The electrodes should be platinized by electrolyzing a dilute solution of platinic chloride (reversing the current so as to platinize both electrodes) then one of dilute sulphuric acid, and washing thoroughly with water. When not in use the cell should be kept filled with distilled water. During the determination the cell should be kept in a constant temperature bath as conductance increases about two per cent with each degree rise in temperature. Connections to the cell are best made through mercury.

A *Resistance Box.*—The dial type is most convenient.

A *Telephone Receiver* (double with headband). An ordinary 2000 ohm headset may be used but for more accurate work a special receiver of lower resistance should be used. Instead of a telephone receiver a galvanometer suitable for use with 110 volt 60 cycle alternating current may be used for most biological work.

A *Slide Wire Resistance.*—An ordinary half meter slide wire resistance may be used. More compact and accurate are the Kohlrausch circular slide wires. Resistance coils at each end of the slide wire are generally desirable. Each should have a resistance four and one-half times that of the slide wire.

A *Source of Alternating Current.*—Alternating current must be used to prevent polarization of the cell. Ordinary 110 volt 60 cycle current may be used with a suitable galvanometer. For the telephone higher frequencies are required. For high accuracy the Vreeland oscillator with 110 volt direct current may be used. A microphone hummer with four dry cells in series may be used. The simplest arrangement consists of one or more dry cells in series with an ordinary telephone buzzer. The buzzer should be placed in a sound proof box some distance from the rest of the apparatus.

A variable air condenser, V. C. compensates for the capacity of the cell C and results in sharper readings.

A *single contact key* or pushbutton. All connections should be made with low resistance wire. The resistance of the wires from the electrode to the bridge should be the same as that of the wires connecting the resistance box with the bridge. If necessary the lead resistance may be corrected for by measuring the resistance with the cell filled with mercury, this resistance then being subtracted from measured resistances.

Determination of Cell Constant.—Measure the resistance of the cell when filled with N/10 potassium chloride. The specific conductance of this solution at 18° is 0.01119. The

Introduce the solution to be tested into the conductivity cell so that it covers both electrodes. Allow to come to a constant temperature. Put on head set and press down sliding contact. A hum is noted showing that a current is passing through the telephone. Introduce resistances at R by removing plugs or turning dials until on moving sliding contact along the wire a point of silence or minimum sound is obtained. Adjust resistances further until the point of minimum sound is somewhere near the center of the slide wire.

The current entering the circuit at the junction of C and R divides part going through C and AD, and the other part through R and DB. At the point of silence the two ends of the slide wire have the same potential and C AD = R BD or C/R = AD/BD. Add up the resistances introduced at R and take the reading of the slide wire. Then X (resistance of cell) : R :: AD : BD or $X = R\frac{AD}{BD}$. With a scale of 100 divisions this becomes $X = R\frac{a}{100 - a}$ or if extension coils are used $X = R\frac{450 + A}{550 - A}$. Thus if the resistance box reads 100 ohms and the slide wire 40, the resistance of the cell $X = 100\frac{40}{60} = 66.7$ ohms. Conductance being the reciprocal of resistance $\left(C = \frac{1}{R}\right)$, the conductance of the cell is 1/66.7 = 0.015 reciprocal ohms or mhos, the mho being the unit of conductance. As cells vary this value must be corrected to obtain the specific conductance of the solution. By *specific conductance* we mean the conductance of a regular cube of the conductor having sides one centimeter long, such as would exist between two platinum electrodes each one centimeter square placed one centimeter apart. To correct for the cell constant K we then have k (specific conductance) = K.c (measured conductance). Or to combine the calculations $k = \frac{K}{R}\cdot\frac{a}{100 - a}$ where a is the slide wire reading. With any given cell a curve may be plotted for $K\cdot\frac{a}{100 - a}$ and the calculation thus simplified.[1]

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cell constant is equal to the measured resistance multiplied by the specific conductance. R1 and R1 are coil resistances each four and one-half times that of the slide wire. Putting them into the circuit (by removing plugs K and K) multiplies the resistance of the slide wire by ten and makes more accurate readings possible.

[1] For tables of values of a/(100-a) see McClendon: Physical Chemistry of Vital Phenomena, Princeton, 1917; or Michaelis: Practical Physical and Colloid Chemistry, London, 1925, or books on physical methods.

CHAPTER II

ENZYMES AND THEIR ACTION[1]

According to the old classification ferments were divided into two classes, the *organized ferments* and the *unorganized ferments*. As organized ferments or true ferments there were grouped such substances as *yeast* and certain *bacteria* which were supposed to act by virtue of vital processes, whereas the unorganized ferments included *salivary amylase* (ptyalin), *gastric protease* (pepsin), *pancreatic protease* (trypsin), etc., which were described as "non-living unorganized substances of a chemical nature." Kühne designated this latter class of substances as *enzymes* (ἐν ζύμῃ—in yeast). This division into organized ferments (true ferments) and unorganized ferments (enzymes) was generally accepted and was practically unquestioned until Buchner overthrew it in the year 1897 by his epoch-making investigations on zymase. Previous to this time many writers had expressed the opinion that the action of the ferment organisms was similar to that of the unorganized ferments or enzymes and that therefore the activity of the former was possibly due to the production of a substance in the cell, which was in nature similar to an enzyme. Investigation after investigation, however, failed to isolate any such principle from an active cell and the exponents of the "vital" theory became strengthened in their belief that certain fermentative processes brought about by living cells could not occur apart from the biological activity of such cells. However, as

[1] For a more extended consideration of enzymes the student is referred to the following sources.

Bayliss.—The Nature of Enzyme Action, Longmans, Green and Co., New York and London.

Cohnheim.—Enzymes, Wiley and Sons, New York.

Duclaux.—Traité de Microbiologie, Masson and Co., Paris.

Effront.—(*a*) Enzymes and their Applications, Translated by Prescott, Wiley and Sons, New York. (*b*) Biochemical Catalysts in Life and Industry. Proteolytic Enzymes, Translated by Prescott and Venable, Wiley and Sons, New York.
Industrial fermentations,

Euler.—Allgemeine Chemie der Enzyme, Bergmann, Wiesbaden. (*b*) Ergebnisse der Physiologie. (*c*) General Chemistry of the Enzymes, Translated by Pope, Wiley and Sons.

Falk.—The Chemistry of Enzyme Actions, The Chemical Catalog Co., New York.

Nord.—Chemical Processes in Fermentations, *Chemical Reviews*, 3, 41, 1926.

Oppenheimer.—Die Fermente und Ihre Wirkungen, Vogel, Leipzig.

Samuely.—Handbuch der Biochemie des Menschen und der Thiere (Oppenheimer), Gustav Fischer, Jena.

Waksman and Davison.—Enzymes: Description, Distribution, Principles, Methods and Applications, Williams and Wilkins, Baltimore, 1926.

Sherman.—Chemistry of Food and Nutrition, Macmillan, New York, Third Edition, 1926.

Wohlgemuth.—Grundriss der Fermentmethoden, Springer, Berlin.

early as 1858, Traube had enunciated, in substance, the principles which were destined to be fundamental in our modern theory of fermentation. He expressed the belief that the yeast cell produced a product in its metabolic activities which had the property of reacting with sugar with the production of carbon dioxide and alcohol, and further that this reaction between the product of the metabolism of the yeast cell and the sugar occurred *without aid from the original cell.* It was not until 1897, however, that this theory was placed upon a firm experimental basis. This was brought about through the efforts of Buchner, who succeeded in isolating from the living yeast cells a substance (zymase) which, when freed from the last trace of organized cellular material, was able to bring about the identical fermentative processes which, up to this time, had been deemed possible only in the presence of the active, living yeast cell.

Buchner's manipulation of the yeast cells consisted in first grinding them with sand and infusorial earth, after which the finely divided material was subjected to great pressure (300 atmospheres) and yielded a liquid which possessed the fermentative activity of the unchanged yeast cell.[1] This liquid contained *zymase*, the principal enzyme of the yeast cell. Later the lactic-acid- and acetic-acid-producing bacteria were subjected by Buchner to treatment similar to that accorded the yeast cells, and the active intracellular enzymes were obtained. Many other instances are on record in which a soluble, active agent has been isolated from ferment cells, with the result that it is pretty well established that all the so-called organized ferments elaborate substances of this character.

Enzymes act by *catalysis* and hence may be termed catalyzers or catalysts. A simple rough definition of a catalyst is "a substance which increases the velocity of a chemical reaction without itself being permanently changed." It is a well-known fact that the velocity of the greater number of chemical reactions may be changed through the presence of some catalyst. For example, take the case of hydrogen peroxide. It spontaneously decomposes slowly into water and oxygen. In the presence of colloidal platinum,[2] however, the decomposition is much accelerated and ceases only when the destruction of the hydrogen peroxide is complete. In the same way sulphur dioxide reacts slowly with oxygen to form sulphur trioxide. If oxides of nitrogen are present they catalyze the reaction so that it proceeds much more rapidly, *e.g.*

$$NO + \tfrac{1}{2}O_2 = NO_2$$
$$SO_2 + NO_2 = SO_3 + NO$$

[1] In later investigations the process was improved by freezing the ground cells with liquid air and finely pulverizing them before applying the pressure.

[2] Produced by the passage of electric sparks between two platinum terminals immersed in distilled water, thus liberating ultra-microscopic particles. (See page 15.)

Without multiplying instances, suffice it to say that there is a close analogy between inorganic catalysts and enzymes, the main point of difference between the enzymes and most of the inorganic catalysts being that the enzymes are colloids. The great majority of enzymes are hydrolytic in character.

We may define an enzyme as *an organic catalyst which is elaborated by an animal or vegetable cell and whose activity is entirely independent of any of the life processes of such a cell.* According to this definition the enzyme *zymase* elaborated by the yeast cell is entirely comparable to the enzyme *pepsin* elaborated by the cells of the stomach mucosa. One is derived from a vegetable cell, the other from an animal cell, yet the activity of neither is dependent upon the integrity of the cell.

Inasmuch as each of the enzymes has an action which is more or less specific in character, and since it is a fairly simple matter, ordinarily, to determine the character of that action, the classification of the enzymes is not attended with very great difficulties. They are ordinarily classified according to the nature of the substrate[1] or according to the type of reaction they bring about. Thus we have various classes of enzymes, such as *amylolytic*,[2] *proteolytic*, *lipolytic*, *glycolytic*, *uricolytic*, *autolytic*, *oxidizing*, *reducing*, *inverting*, *protein-coagulating*, *deamidizing*, etc. In every instance the class name indicates the individual type of enzymatic activity which the enzymes included in that class are capable of accomplishing. For example, amylolytic enzymes facilitate the hydrolysis of starch (amylum) and related substances, lipolytic enzymes facilitate the hydrolysis of fats (λιπος), whereas through the agency of uricolytic enzymes uric acid is broken down. There is a tendency, at the present time, to harmonize the nomenclature of the enzymes by the use of the termination *-ase*. According to this system of nomenclature, all starch-transforming enzymes, or so-called amylolytic enzymes, are called *amylases;* all fat-splitting enzymes are called *lipases*, etc. Thus *ptyalin*, the amylolytic enzyme of the saliva, would be termed *salivary* amylase in order to distinguish it from *pancreatic* amylase (amylopsin) and *vegetable* amylases (diastase, etc.). According to the same system, the fat-splitting enzyme of the gastric juice would be termed *gastric* lipase to differentiate it from *pancreatic* lipase (steapsin), the fat-splitting enzyme of the pancreatic juice.

Defensive (*protective*) *enzymes* are those believed to be manufactured by certain cells (perhaps the leucocytes) and passed into the

[1] Substance acted upon. See Lippmann: *Ber. d. Deutsch. Chem. Ges.*, 36, 331, 1903.

[2] Armstrong suggests the use of the termination "clastic" instead of "lytic." He calls attention to the fact that *amylolytic*, in analogy with *electrolytic*, means "decomposition by means of starch" and is therefore a misnomer. He suggests the use of *amyloclastic*, *proteoclastic*, etc.

circulating blood in order to digest any foreign material of endogenous or exogenous origin that may have found its way into the circulation. The most important defensive enzymes are proteolytic enzymes. Abderhalden[1] claims that the parenteral introduction of any foreign protein into the animal body will be followed by the appearance in the blood of a defensive enzyme capable of digesting that protein. He also claims that in pregnancy the passage into the blood of protein material in the form of cells and fragments of chorionic villi will cause the appearance in the blood of a defensive proteolytic enzyme capable of digesting placenta protein. The *Abderhalden reaction* for pregnancy is based upon this hypothesis. The reaction has been widely employed and much has been said both for and against its accuracy.[2] Modifications of the reaction have been suggested as aids in the diagnosis of various disorders, *e.g.*, cancer, tuberculosis, dementia præcox, etc. These are also of doubtful value. Later work has also thrown much doubt upon the original contention of Abderhalden as to the formation of protective enzymes following the parenteral introduction of protein, carbohydrates and other substances. Abderhalden, however, maintains that his original contention is correct.[3]

Our knowledge regarding the distribution of enzymes has been wonderfully broadened in recent years. Up to within a few years, the real scientific information as to the enzymes of the animal organism, for example, was limited, in the main, to a rather crude understanding of the enzymes intimately connected with the main digestive functions of the organism. We now have occasion to believe that enzymes are doubtless present in every animal cell and are actively associated with all vital phenomena. A preëminent example of such cellular activity is the liver cell with its reputed complement of 15 to 20 or more enzymes.

Attempts to isolate pure enzymes have not usually been successful owing to the ease of their destruction by heat, precipitation etc. Purified preparations have been obtained in certain cases, notably pepsin,[4] malt amylase,[5] pancreatic amylase, (see page 56) and lipase.[6] By the products of their hydrolysis, their solubility relations and re-

[1] *Abwehrfermente des tierischen Organismus*, 5th Ed., 1915, Springer, Berlin.

[2] Bronfenbrenner: *Jour. Am. Med. Assn.*, 65, 1268, 1915. Van Slyke: *New York Med. Jour.*, 103, 219, 1916. Taylor: *Jour. Biol. Chem.*, 22, 59, 1915. Hulton: *Jour. Biol. Chem.*, 25, 163, 1916. Kuriyama: *Jour. Biol. Chem.*, 25, 534, 1916. Lüttge and v. Mertz: *Münch. med. Woch.*, 71, 576, 1924. Lahmann: *ibid.*, 72, 1507, 1925. Herzfeld, Kretschmer and Wittenberg: *Klin. Woch.*, 4, 2302, 1925.

[3] Abderhalden: *Correspondenz-Blatt für Schweizer Aerzte*, 47, 1745, 1917; *Med. Klinik* 17, 1453, 1921; Die Abderhaldensche Reaktion, Springer, 1922.

[4] Pekelharing, *Z. physiol. Chem.* 35, 8, 1902. Aldrich, *J.Biol. Chem.* 23, 339, 1915. Davis and Merker, *J. Amer. Chem. Soc.* 41, 221, 1919.

[5] Osborne, *J. Amer. Chem. Soc.*, 17, 587, 1895; 18, 536, 1896.

[6] Falk and Sugiura, *J. Am. Chem. Soc.* 37, 217, 1915.

CLASSIFICATION OF ENZYMES

Name and Class	Distribution	Substrate	End-products
Carbohydrases		Carbohydrates	
1. *Amylases*		Starch, dextrin, etc.	
(a) *Pancreatic* (amylopsin)	Pancreatic juice	Starch, dextrin, etc.	Maltose.
(b) *Salivary* (ptyalin)	Saliva	Starch, dextrin, etc.	Maltose.
(c) *Vegetable*	Malt, rice fungus, etc.	Starch, dextrin, etc.	Maltose.
2. Glycogenase	Liver, muscles?	Glycogen	Dextrin and maltose (glucose?)
3. Inulase	Fungi, other plants	Inulin	Fructose.
4. *Lactase*	Intestinal juice and mucosa.	Lactose	Glucose and galactose.
5. *Maltase*	Blood serum, liver, saliva, pancreatic and intestinal juices and lymph.	Maltose	Glucose.
6. *Sucrase* (invertase)	Intestinal juice and mucosa.	Sucrose	Glucose and fructose.
7. *Zymase*	Yeast	Sugars	Alcohol, CO_2, etc.
Carboxylase	Yeast	COOH group of aliphatic acids.	Carbon dioxide.
Deaminases		Amino compounds	
1. Adenase	Animal tissues	Adenine	Hypoxanthine.
2. Arginase	Intestine, liver, kidney, spleen, etc.	Arginine	Ornithine and urea.
3. Guanase	Animal tissues	Guanine	Xanthine.
4. *Urease*	Micrococcus ureæ, soy bean, jack bean, etc.	Urea	Carbon dioxide and ammonia.
Glucosidases		Glucosides (amygdalin and others).	
1. Emulsin	Plants	β-glucosides	Glucose, etc.
2. Invertase	Yeast, etc.	α-glucosides	Glucose, etc.
Lipases		Fats	
1. Autolytic	Animal tissues	Fats	Fatty acid and glycerol
2. *Pancreatic* (steapsin)	Pancreatic juice	Fats	Fatty acid and glycerol.
3. Vegetable	Castor bean, etc.	Fats	Fatty acid and glycerol.
Nucleases		Nucleic acid and derivatives	
1. Nucleicacidase	Intestinal mucosa and juice, other tissues.	Nucleic acid	Nucleotides.
2. Nucleotidase	Intestinal mucosa and juice, other tissues.	Nucleotides	Phosphoric acid and nucleosides.
3. Nucleosidase	Tissues	Nucleosides	Carbohydrate and bases.
Oxidases.			
1. *Catalase*	Plant and animal tissues.	Hydrogen peroxide	Molecular oxygen.
2. Laccase	Lac tree, fungi, etc.	Polyhydric para-phenols as hydroquinol and pyrogallol.	Oxidation products.
3. *Peroxidase*	Plant and animal tissues.	Peroxides	Active oxygen and oxidation products.
4. Purine-oxidases.		Purines.	
(a) Hypoxanthine oxidase.	Animal tissues	Hypoxanthine	Xanthine.
(b) Uricase	Animal tissues	Uric acid	Allantoin.
(c) Xanthine oxidase.	Animal tissues	Xanthine	Uric acid.
5. Tyrosinase	Plant and animal tissues.	Tyrosine	Homogentisic acid, etc.
Peptases		Polypeptides	
1. *Erepsin*	Intestinal mucosa and juice, other tissues.	Peptides, and peptones.	Simpler peptides and amino acids.
Phytase	Rice bran, liver, blood.	Phytin	Inositol and phosphoric acid.
Glycerophosphatase	Intestinal mucosa etc.	Glycerophosphoric acid.	Phosphoric acid and glycerol.
Hexosephosphatase	Intestinal mucosa etc.	Hexosephosphoric acid.	Phosphoric acid and hexose.

CLASSIFICATION OF ENZYMES.—*Continued*

Name and Class	Distribution	Substrate	End-products
Proteases.		Proteins	
1. *Coagulases*		Proteins in solution	
(a) *Rennin* (gastric)	Gastric juice	Casein	Paracasein.
(b) Rennin (pancreatic)	Pancreatic juice	Casein	Paracasein.
(c) *Thrombin*	Blood	Fibrinogen	Fibrin.
2. *Pepsin* (acid-protease)	Gastric juice	Proteins	Proteoses, peptones, and peptides.
3. *Trypsin* (alkali-protease)	Pancreatic juice	Proteins	Proteoses, peptones, and peptides.
4. Vegetable proteases.			
(a) Bromelin	Pineapple	Proteins	Proteoses, peptones, etc
(b) Papain (papayotin).	Pawpaw	Proteins	Proteoses, peptones, etc
Purinases (see Purine Oxidases and Purine Deaminases).			

sponse to protein color reactions these have been shown to be protein in nature. Since they possess colloidal properties, the criterion of purity, crystallization, has been inapplicable. Success in the crystallization of an enzyme has recently been reported by Sumner,[1] who by refrigeration of an acetone extract of jack bean meal (see page 64 prepared sharply defined octahedral crystals believed to be identical with urease (see Fig. 12A). The product is protein in nature and is

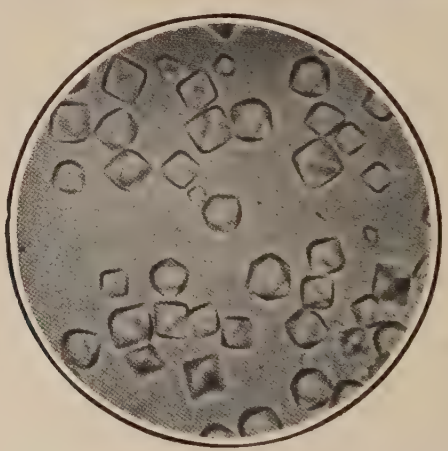


FIG. 12A.—UREASE CRYSTALS ×728. (*Sumner: Jour. Biol. Chem.*, 69, 435, 1926.)

apparently a globulin. In concentrated solution this protein is extremely active, one gm. producing 100 gm. of ammonia nitrogen from urea-phosphate solution in five minutes at 20°C. Determination of the activity of the crystals has thus far been unsuccessful due to apparent instability of the enzyme at great dilution.

We may summarize some of the properties of the great body of enzymes as follows: Enzymes are soluble in *dilute* glycerol, sodium chloride

[1] Sumner, *Jour. Biol. Chem.*, 69, 435, 1926. This paper appeared while the present edition was in press.

solution, *dilute* alcohol, and water, and precipitable by ammonium sulphate and *strong* alcohol. Their presence may be proven from the nature of the end-products of their action and not through the agency of any chemical test. They are *colloidal* and *non-diffusible*, and occur closely associated with protein material with which they generally possess many properties in common. Each enzyme shows the greatest activity at a certain temperature called the *optimum* temperature, there is also a *minimum* and a *maximum* temperature for each specific enzyme. Their action is inhibited by sufficiently lowering the temperature, although some activity may be shown at 0°C. or even at lower temperatures and freezing does not, in most cases, permanently injure enzymes. Most enzymes, if in solution, are entirely destroyed by subjecting them to a temperature of 70°–100°C. The best known enzymes, whether derived from warm-blooded or cold-blooded animals, are most active between 35°–45°C. The nature of the surrounding media alters the velocity of the enzymatic action, some enzymes being more active in acid solution whereas others require an alkaline fluid.

Many of the more important enzymes do not occur preformed within the cell, but are present in the form of a *zymogen* or mother-substance. In order to yield the active enzyme this zymogen must be transformed in a certain specific manner and by a certain specific substance. This transformation of the inactive zymogen into the active enzyme is termed *activation*. For instance, the zymogen of the enzyme pepsin of the gastric juice, termed *pepsinogen*, is activated by the *hydrochloric acid* secreted by the gastric cells (see page 203), whereas the activation of the *trypsinogen* of the pancreatic juice is brought about by a substance termed *enterokinase*[1] (Chapter XII). These are examples of many well-known activation processes going on continually within the animal organism. The agency which is instrumental in activating a zymogen is generally termed a *zymo-exciter* or a *kinase*. In the cases cited hydrochloric acid would be termed a zymo-exciter and enterokinase would be termed a kinase.

After filtering yeast juice, prepared by the Buchner process (see page 42), through a Martin gelatin filter, Harden and Young showed that the colloids left behind and the filtrate were both inactive fermentatively. Upon treating the colloid material (enzyme) with some of the filtrate, however, the mixture was shown to be able to bring about pronounced fermentation. It is believed that a *co-enzyme* present in the filtrate was the efficient agent in the transformation of the inactive enzyme. It is necessary to make frequent renewals of the co-enzyme in order to maintain continuous fermentation. It was further shown that this co-enzyme, in addition to being diffusible, was not destroyed

[1] According to Delezenne, trypsinogen may be rapidly activated by soluble calcium salts.

by boiling and that it disappeared from yeast juice when this latter was fermented or allowed to undergo autolysis. The exact nature of this co-enzyme of zymase is unknown. The co-enzyme action, in this case, is probably dependent upon the presence of two individual agencies, one of which is *phosphates*. The formation of a glucose phosphate is apparently a necessary preliminary to the oxidation of the sugar by yeast or by animal cells.

It has been shown by Loevenhart that the property of acting as a pancreatic lipase co-enzyme is vested in *bile salts*, and Magnus has further shown that the synthetic salts are as efficient in this regard as the natural ones. A few other instances of co-enzyme demonstrations have been reported.

Electrolytes are very important factors in facilitating or inhibiting enzyme action.[1] For example, the Cl ion in proper amount facilitates the action of amylases.[2] In fact the presence of the Cl or Br ion is apparently absolutely essential to the activity of pancreatic amylase, inasmuch as dialysis renders this enzyme inactive, the activity returning on the addition of sodium chloride.[3] The acidity or hydrogen ion concentration of the solution also exerts much influence on the activity of enzymes. The presence of certain amino acids, *e.g.* tryptophane and glycocoll has been shown to facilitate enzyme action (amylase, etc.). It has been demonstrated in the case of certain enzymes, at least, that the continuous vibration or shaking of their solutions tends to produce a destruction of the enzyme. Ultraviolet light also has a destructive action on enzymes.[4]

The so-called "specificity" of enzyme action is an interesting and important fact. That enzymes are very specific as to the character of the *substrate*, or substance acted upon, is well known. Emil Fischer investigated this problem of specificity extensively in connection with the fermentation of sugars and reached the conclusion that enzymes, with the possible exception of certain oxidases, can act only upon such substances as have a specific stereo-isomeric relationship to themselves. He considers that the enzyme and its substrate must have an interrelation, *such as the key has to the lock*, or the reaction does not occur. Fischer was able to predict, in certain definite cases, from a knowledge of the constitution and stereo-chemical relationships of a substance, whether or not it would be acted upon by a certain enzyme. An application of this specificity of enzyme action may be seen in the well-known facts that certain enzymes act on carbohydrates, others on fats, and

[1] For literature, see Kendall and Sherman: *Jour. Am. Chem. Soc.*, 32, 1087, 1910.
[2] Wohlgemuth: *Biochemische Zeitschrift*, 9, 10, 1908.
[3] Bierry: *ibid.*, 40, 357, 1912.
[4] Hussey and Thompson: *Jour. Gen. Physiol.*, 9, 217, 1925.

others on protein; and, moreover, that the group of those which transform carbohydrates, for example, is further subdivided into specific enzymes each of which has the power of acting alone upon some one sugar.

It has been conclusively shown, in the case of certain enzymes,[1] at least, that their action is a *reversible* one and is, in all its main features, directly analogous to the reversible reactions produced by chemical means. For instance, in the saponification of ethyl-butyrate by means of pancreatic lipase, it has been shown that upon the formation of the end-products of the reaction, *i.e.*, butyric acid and ethyl alcohol, there is reversion[2] and the reaction is stationary. This does not mean there are no chemical changes going on, but simply indicates that chemical *equilibrium* has been established, and that the change in one direction is counterbalanced by the change in the opposite direction. Pancreatic lipase was one of the first enzymes to have the reversibility of its reaction clearly demonstrated.[3] A knowledge of the fact that lipase possesses this reversibility of action is of extreme physiological importance and aids us materially in the explanation of the processes involved in the digestion, absorption, and deposition of fats in the animal organism (see page 246). The reversibility of peptic and tryptic activity has been demonstrated by Wasteneys and Borsook[4] who were able under optimal conditions to resynthesize 39 per cent of a peptic digest of egg albumin. Under ordinary conditions, where enzyme action takes place in the presence of an excess of water, these reactions reach an equilibrium point near the stage of completion.

Euler[5] claims that enzymatic *cleavage* and *synthesis* are often brought about by two different components of an enzyme preparation. He would indicate this fact by giving the termination *-ese* to those enzymes exerting a synthetic function. For example, the enzyme which catalyzes the formation of nitriles Euler would call nitril*ese* in distinction from nitril*ase* which splits nitriles. He would further designate as phosphat*ese* the enzyme which builds up phosphoric acid esters of carbohydrates in distinction from phosphat*ase* which causes their cleavage. In the same way he would differentiate the lipolytic enzymes into lip*ases* and lip*eses*.

In respect to many enzymes it has been found that the law governing the action of inorganic catalyzers is directly applicable, *i.e.*, that the *intensity is almost directly proportional to the concentration of the*

[1] This is probably a general condition.

[2] The re-synthesis of ethyl-butyrate from its hydrolysis products. This may be indicated thus:

$$\underset{\text{Ethyl-butyrate.}}{C_3H_7COO.C_2H_5} + H_2O \leftrightarrows \underset{\text{Butyric acid.}}{C_3H_7COOH} + \underset{\text{Ethyl alcohol.}}{C_2H_5OH}.$$

[3] This principle was *first* demonstrated in connection with the enzyme maltase (see p. 108).

[4] Wasteneys and Borsook: *Jour. Biol. Chem.*, 62, 15, 633, 675, 1924–25; 63, 575, 1925.

[5] Euler: *Zeitschrift für physiologische Chemie*, 74, 13, 1911.

enzyme. In the case of enzymes, however, there is a difference in that a maximum intensity is soon reached and that subsequent concentration of the enzyme is productive of no further increase in intensity. Enzymes which have been shown to obey this *linear law* are lipase, sucrase, rennin, and trypsin. In *certain instances*, where this law of direct proportionality between the intensity of action and the concentration of enzymes does not hold, it has been found that the *Schütz-Borissow law*, first experimentally demonstrated by E. Schütz, was applicable. This is to the effect that the intensity is directly proportional to the square root of the concentration, or conversely, that *the relative concentrations of enzyme preparations are directly proportional to the squares of the intensities.*[1] This Schütz-Borissow law is not generally applicable.

Northrop[2] has applied the classical theories of chemistry to the kinetics of peptic and tryptic activity and concludes that the reaction takes place with the ionized portion of the substrate. There is possibly a reversible combination of enzyme with the products of digestion. If the substrate is in the solid phase, adsorption effects enter in.

It has been shown that there are certain substances which possess the property of directly inhibiting or preventing the action of a catalyzer. These are called *anti-catalyzers* or paralyzers and have been compared with the *anti-toxins*. Related to this class of anti-catalytic agents stand the *anti-enzymes*. The first anti-enzyme to be reported was the *anti-rennin* of Morgenroth. This was produced by injecting into an animal increasing doses of rennet solution, whereupon an "anti" substance was subsequently found both in the serum and in the milk, which prevented the enzyme rennin from exerting its normal activity in the presence of casein. In other words, *anti-rennin* had been formed in the serum of the animal,[3] through the repeated injections of rennet solution. Since the discovery of this anti-enzyme, anti-bodies have been demonstrated for pepsin, trypsin, lipase, urease, amylase, laccase, tyrosinase, emulsin, papain, and thrombin. According to Weinland, the reason why the stomach does not digest itself is, that during life there is present in the mucous membrane of the stomach an *anti-enzyme* (*anti-pepsin*) which has the property of inhibiting the action of pepsin. A similar substance (*anti-trypsin*) is present in the intestinal mucosa as well as in the tissues of various intestinal worms. It is probable that among the substances commonly classified as anti-enzymes are included inhibitory agents of widely differing characters.

[1] An excellent review of the kinetics of enzyme action is to be found in Bayliss' "Principles of General Physiology," 1924.

[2] Northrop: *Jour. Gen. Physiol.*, 2, 113, 1919; 5, 263, 1922.

[3] Serum is normally *anti-tryptic.*

The investigations of Ehrlich[1] and of Neuberg[2] have served to cause a complete revision of our ideas regarding yeast fermentation. Ehrlich, for example, has shown that *yeast will liberate ammonia from amino acids* and leave behind a non-nitrogenous complex. Among these complexes amyl alcohol, succinic acid and others may be mentioned. Thus, *amyl alcohol* results from the fermentation of leucine, whereas ethyl alcohol results from the fermentation of sugar. Neuberg has demonstrated the presence in the yeast of an enzyme termed *carboxylase* which has the property of *splitting off carbon dioxide from the carboxyl group of amino and other aliphatic acids.* The findings mentioned above constitute the basis for much important work on so-called "sugar-free fermentation."

Oxidation and Reduction Systems.—We have somewhat more information as to the nature and mode of action of certain oxidase systems of plant and animal cells. Most if not all of the energy of living matter is derived from oxidative processes. The substances principally utilized are carbohydrates, fats, and proteins which do not readily undergo oxidation in the air at ordinary temperatures. The processes involved are thus of great interest although not yet well understood[3]. Oxidations in animal tissues are most readily studied by means of a microrespirometer (for diagram illustrating the principle see Fig. 13).[4]

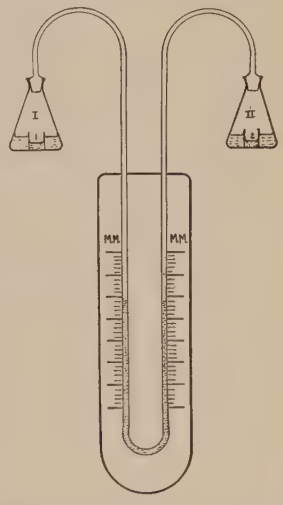


FIG. 13.—DIAGRAM OF MICRORESPIROMETER, FOR STUDYING OXYGEN CONSUMPTION OF TISSUES.

[1] Ehrlich: *Biochemische Zeitschrift*, 36, 477, 1911.

[2] Neuberg and collaborators: *Biochemische Zeitschrift*, 31, 170; 32, 323; 36 (60, 68, and 76), 1911.

[3] For more detailed discussions see Evans: Recent Advances in Physiology, Philadelphia, 1926 and numerous papers by Hopkins, Thurlow, Tunnicliffe, Dixon and others in the *Biochemical Journal*. For other forms of the appa: ratus see Warburg: *Biochem. Zeit.* 142, 317, 1923 and Koehler: *Jour. Biol. Chem.* 63, 475, 1925. For studies on oxidation reduction reactions using electrometric methods see Clark, Cohen, and Gibbs: *Public Health Reports*, Vol. 40, Pt. 1, p. 1131, 1925, which includes a bibliography.

[4] Flask I contains the minced tissue suspended in some suitable fluid such as Ringer's solution. Flask II contains an equal amount of Ringer's solution but no tissue. The flasks are connected to a Barcroft type manometer of small bore containing kerosene. The small vessels 1 and 2 contain small amounts of potassium hydroxide solution. The flasks are kept in a constant temperature bath and the air may be replaced with oxygen. The manometer should at the start show the fluid at the same level in each arm. A shaking device which moves the flasks backward and forward in a horizontal direction is essential. As oxygen is absorbed by the tissue in flask I the fluid in the arm of the barometer attached to it rises and the level in the other arm falls. A series of readings of the manometer is taken during the course of the experiment and from them the oxygen consumption of the tissues may be calculated. The alkali in the small vessels absorbs any carbon dioxide liberated. By running the experiments with and without the presence of alkali the amount of CO_2 given off may also be estimated and the ratio of the CO_2 given off to oxygen consumed (the respiratory quotient) may be calculated. Another method by which tissue oxida ons may be studied is that of Thunberg (see p. 62). To the test mixtures in glass stoppered test tubes are added definite amounts of methylene blue. The tubes are then

Wherever an oxidation occurs there must be a simultaneous reduction. Thus when hydrogen combines with oxygen to form water the hydrogen may be said to be oxidized and the oxygen to be reduced. The essential feature of all oxidation processes is the transfer of negative electrons from the substance oxidized to the substance reduced whereby the number of negative valences of the former is reduced or positive valences increased. The reaction of ferric chloride and potassium iodide involves a transfer of electrons from the iodine atom to the iron atom so the iodide is said to be oxidized and the iron reduced.

$$Fe^{+++} + 3Cl^- + K^+ + I^- \rightarrow Fe^{++} + 3Cl^- + K^+ + I$$

Hydrogen peroxide especially in the presence of ferrous compounds brings about many oxidations similar to those occurring in the animal body. It is desirable to know therefore whether hydrogen peroxide and other peroxides occur in plant and animal cells. If an aqueous potato extract be prepared and treated with guaiac solution the latter will turn blue indicating that it has been oxidized by active oxygen present. If kept out of contact with air for some time and then guaiac added without admitting oxygen no blue oxidation product of the guaiac will be formed. If air is admitted again however oxidation takes place. By alcoholic precipitation of potato extracts a substance can be obtained which will not oxidize guaiac even in contact with air but will do so if hydrogen peroxide or certain other peroxides are present. This substance is a catalyzer or enzyme and is called a *peroxidase*. The other substance present in the potato which can take up oxygen from the air to form a peroxide has been called an *oxygenase* or *autoxidizable substance*. The combination of peroxidase and oxygenase is called an *oxidase system*. In the potato the autoxidizable substance appears to be of a lecithin-like character. There is reason to believe that unsaturated fatty acids particularly linolenic acid may possess similar properties. The oxidation of linolenic acid is catalyzed by minute amounts of salts of iron and other metals. It has been suggested that a peroxide grouping may be formed at one of the double bonds of the fatty acid as follows.

$$-CH = CH- \rightarrow -CHOH{\cdot}CHOH- \rightarrow \begin{matrix} H & H \\ | & | \\ -C & -C- \\ | & | \\ O & -O \end{matrix}$$

evacuated to get rid of free oxygen and the time required for methylene blue to be reduced and decolorized is measured. This times is a measure of the rapidity with which oxidation processes are simultaneously occurring in the system. McNair[1] has suggested the use of the Van Slyke amino-nitrogen apparatus for the study of oxidases.

[1] McNair: *Jour. Inf. Dis.*, 20, 485, 1917.

Hopkins has isolated from yeast and muscle an autoxidizable substance apparently present in all living cells which he has named *glutathione*. This in its reduced form is a compound (dipeptide) of glutamic acid and cysteine having the formula:

$$\begin{array}{l} \text{COOH}\cdot\text{CH}(\text{NH}_2)\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CO}\cdot\text{NH}\cdot\text{CH.COOH} \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad | \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad \text{CHSH} \end{array}$$

The active group is the $-\text{SH}$ group and the compound for convenience may be referred to as GSH. The substance is readily oxidized with the loss of one hydrogen atom from each of two molecules which then combine to form a compound whose structure is indicated by GS—SG. In the presence of free oxygen (aerobic conditions) the action of glutathione may be as follows, (active hydrogen being indicated by $\text{H}^{\cdot}$)

$$2\text{GSH} \rightarrow \text{GS—SG} + 2\text{H}^{\cdot}$$
$$2\text{H}^{\cdot} + \text{O}_2 \rightarrow \text{H}_2\text{O}_2$$

In the presence of peroxidase active oxygen would be formed:

$$\text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \text{O}^{\cdot}$$

Thus glutathione and peroxidase form an oxidase system.

Besides oxidation by means of active oxygen we may have oxidation by dehydrogenation. An example of this is the oxidation of carbon monoxide in the absence of free oxygen but in the presence of a catalyst such as palladium. The reaction is believed to be first a hydration and than a dehydrogenation thus:

$$\text{CO} + \text{H}_2\text{O} \rightarrow \text{HCOOH} \rightarrow \text{CO}_2 + 2\text{H}^{\cdot}$$

In order that the reaction shall take place we need also a substance which will take up the active hydrogen formed. Such a substance is called a *hydrogen acceptor*. Methylene blue is one substance that can be used. The reaction is then completed as follows:

$$\text{M (methylene blue)} + 2\text{H}^{\cdot} \rightarrow \text{MH}_2 \text{ (methylene white)}.$$

Similar reactions occur in living tissues. Thus Thunberg has shown that succinic acid is dehydrogenated to form fumaric acid the enzyme producing the active H being called a dehydrogenase. The reactions are $\begin{array}{l}\text{CH}_2\text{COOH} \\ | \\ \text{CH}_2\text{COOH}\end{array} \rightarrow \begin{array}{l}\text{CHCOOH} \\ \| \\ \text{CHCOOH}\end{array} + 2\text{H}^{\cdot}$ and $\text{M} + 2\text{H}^{\cdot} \rightarrow \text{MH}_2$.

Not only may oxidations be brought about by activation of either oxygen or hydrogen but both processes may occur simultaneously. In

the presence of oxygen this may act as a hydrogen acceptor to form H_2O_2 and the latter by decomposition may give active oxygen for a second oxidation. In the absence of oxygen (anaerobic conditions) the oxidation of a substance R in the presence of methylene blue M may be represented as follows.

$$RH_2 \rightarrow R + 2H^{\cdot} \qquad M + 2H^{\cdot} \rightarrow MH_2$$

In the presence of oxygen (aerobic conditions) we may have:

(*a*) $RH_2 \rightarrow R + 2H^{\cdot}$	Oxidation by dehydrogenase.
(*b*) $2H^{\cdot} + O_2 \rightarrow H_2O_2$	Peroxide formation.
(*c*) $H_2O_2 \rightarrow H_2O + O^{\cdot}$	Peroxidase action.
(*d*) $R_1 + O^{\cdot} \rightarrow R_1O$	Oxidation of second substance.
(*e*) $2H_2O_2 \rightarrow 2H_2O + O_2$	Catalase producing molecular oxygen.

This agrees with the observations of Thurlow using the system hypoxanthine, xanthine oxidase, peroxidase, and nitrite. The hypoxanthine may be oxidized by reaction (*a*) and the nitrite by reaction (*d*). If catalase is present reaction (*e*) takes place instead of reaction (*c*) and molecular oxygen is produced. The catalase prevents accumulation of peroxide which is destructive to peroxidase and this may be its chief function.

Iron is believed to catalyze the formation of active oxygen in the peroxidase system but not the formation of active hydrogen in the dehydrogenase system. Cyanide inhibits the former type of oxidation but not the latter. It may inactivate the iron salt. Hemoglobin is a peroxidase according to this theory.

The mode of action of glutathione is not completely understood. It may act as a hydrogen acceptor:

$$GS—SG + 2H^{\cdot} \rightarrow 2GSH.$$

The oxidized form may also be reduced by certain thermostable tissue constituents present in boiled, alcohol extracted tissues. The reduced form may then give up its hydrogen to another hydrogen acceptor such as methylene blue. Glutathione with thermostable tissue residues thus forms a thermostable oxidation-reduction system. The tissue residues contain $-SH$ groups the presence of which in reduced form is essential for the success of the system. There is evidence that these groups may themselves be reduced by still other protoplasmic groups. It has already been pointed out that glutathione with peroxidase may form another type of oxidase system.

EXPERIMENTS ON ENZYMES, ANTI-ENZYMES AND INORGANIC CATALYSTS

A. Experiment on Inorganic Catalysts

Catalysis of Decomposition of Hydrogen Peroxide.—Transfer 5 c.c. portions of hydrogen peroxide to each of four test tubes. Add to one a very small amount of finely divided metallic platinum,[1] to the second a small amount of powdered magnetite (Fe_3O_4) and to the third a similar amount of ordinary ferric oxide or hematite (Fe_2O_3). Note any evolution of gas. After a few minutes add a few drops of an alcoholic solution of guaiac. This is oxidized to a blue color by active oxygen. Which tubes show this reaction?

B. Experiments on Enzymes[2]

I. AMYLASES

1. Demonstration of Salivary Amylase.[3]—To 25 c.c. of a 1 per cent starch paste in a small beaker, add 5 drops of saliva and stir thoroughly. At intervals of a minute remove a drop of the solution to one of the depressions of a test-tablet and test by the iodine test.[4] If the blue color with iodine still forms after five minutes, add another 5 drops of saliva.

The opalescence of the starch solution should soon disappear, indicating the formation of *soluble starch* (*amidulin*) which gives a blue color with iodine. This body should soon be transformed into *erythrodextrin* which gives a red color with iodine, and this, in turn, should pass into *achroodextrin* which gives *no color* with iodine. This point is called the *achromic point*. When this point is reached test by Benedict's test[5] to show the production of a reducing substance (maltose). A positive Benedict's test may be obtained while the solution still reacts red with iodine, inasmuch as some sugar is formed from the soluble starch coincidently with the formation of the erythrodextrin. If starch paste be boiled with a small amount of acid a similar decomposition takes place due to the catalytic action of the hydrogen ion. For further discussion of the transformation of starch see pages 92 and 106.

[1] Use a solution of colloidal platinum (see p. 15) or a powder prepared by dipping filter paper in platinic chloride solution and igniting in a crucible. Welo and Baudisch (*Jour. Biol. Chem.* 65, 215, 1925) have shown that magnetite produces active oxygen even when heated to 330°C. although it now has the same composition (Fe_2O_3) as it has when heated still further to 550°C. and the latter product sets free molecular but not active oxygen. Ferrous hydroxide also catalyzes a rapid decomposition of peroxide.

[2] If it is deemed advisable by the instructor to give all the practical work upon enzymes at this point in the course, additional experiments will be found in Chapters IV, VII, VIII, XI and XII.

[3] For a discussion of this enzyme see p. 106.

[4] See p. 93.

[5] See p. 76.

2. Demonstration of Pancreatic Amylase.[1]—Proceed exactly as indicated above in the Demonstration of Salivary Amylase except that the saliva is replaced by 5 c.c. of pancreatic extract prepared as described on p. 259.[2] Pancreatic amylase transforms the starch in a manner entirely analogous to the transformation resulting from the action of salivary amylase.

3. Preparation of Vegetable Amylase.—Extract finely ground malt with water, filter and subject the filtrate to alcoholic fermentation by means of yeast. When fermentation is complete filter off the yeast and precipitate the amylase from the filtrate by the addition of alcohol. The precipitate may be filtered off and obtained in the form of a fine white powder.

A purer preparation[3] is obtained if the solution is dialyzed against water at about 10°C. (in the ice-box) for 24 hours, filtered and precipitated with alcohol or acetone. First alcohol or acetone to make a 50 per cent solution is added, the precipitate thus formed being rejected, while the precipitate formed on the addition of sufficient alcohol or acetone to make a final concentration of 65–70 per cent is preserved, and dried in a vacuum desiccator at a low temperature. By slight modifications of the above procedure Sherman and collaborators[3] have prepared the most potent amylase thus far reported. This preparation, in 30 minutes at 40° split 20,000 times its weight of starch and formed 10,000 times its weight of maltose. In long experiments it digested four million times its weight of starch and formed nearly three million times its weight of maltose. The enzyme was active at a dilution of 1 : 100,000,000.

4. Demonstration of Vegetable Amylase.—This enzyme may be demonstrated according to the directions given under Demonstration of Salivary Amylase, page 55, with the exception that the saliva used in that experiment is replaced by an aqueous solution of the vegetable amylase powder prepared as described above.[4]

II. PROTEASES

1. Preparation of Gastric Protease.[5]**—Treat the finely comminuted mucosa of a pig's stomach with 0.4 per cent hydrochloric acid and extract at 38°C. for 24–48 hours. The filtrate from this mixture constitutes a very satisfactory acid extract of gastric protease (see page 210).**

2. Demonstration of Gastric Protease.—Introduce some protein material (fibrin, coagulated egg-white, or washed lean beef) into the acid extract of gastric

[1] For a discussion of this enzyme see p. 258.

[2] Commercial preparations of pancreatic amylase may be substituted for the pancreatic extract.

[3] Sherman and Schlesinger: *J. Am. Ch. Soc.*, 33, 1195, 1911; 34, 1104, 1912; 37, 643 and 1305, 1915; Sherman: *Proc. Nat. Acad. Sci.*, 9, 81, 1923 and Sherman, Caldwell and Adams: *Proc. Soc. Expt. Biol. & Med.*, 23, 413, 1926.

[4] If desired the *first* aqueous extract of the original malt may be used in this demonstration. Commercial *taka-diastase* may also be employed.

[5] Also called *pepsin*, *pepsase*, *gastric protease* and *acid protease*. For a discussion of this enzyme see p. 203.

protease prepared as above described,[1] add an equal volume of 0.4 per cent hydrochloric acid and place the mixture at 38°C. for 2–3 days. Identify the products of digestion according to directions given on page 211.

Carmine-fibrin may also be used in this test. This is prepared by running fibrin through a meat chopper washing carefully and placing in a ½ per cent ammoniacal carmine solution (very little excess ammonia should be present) until the maximum coloration of the fibrin (a dark red) is obtained. The fibrin is then washed in water and water acidified with acetic acid. It is preserved under glycerol.

To 15 c.c. of the solution to be tested add a small amount of the carmine fibrin and allow to digest at *room temperature*. Digestion will be shown by the setting free of carmine with coloration of the solution. This is a delicate test for pepsin. A control should be run using acid of same strength as that of enzyme solution tested.

3. Preparation of Pancreatic Protease.[2]—A satisfactory extract of this enzyme may be made from the pancreas of a pig or sheep according to the directions given on page 259.

4. Demonstration of Pancreatic Protease.—Into an alkaline extract of pancreatic protease,[3] prepared as directed on page 259, introduce some fibrin, coagulated egg-white or lean beef and place the mixture at 38°C. for 2–5 days.[4] At the end of that period separate and identify the end-products of the action of pancreatic protease according to the directions given on page 259.

Congo-red fibrin may be used in this test. This may be prepared by placing fibrin in faintly alkaline congo-red solution and heating to 80°C. The fibrin is then washed. A small amount of this colored fibrin is placed in the slightly alkaline solution of the enzyme. Digestion is shown by a red coloration of the solution due to setting free of congo red.

5. Demonstration of a Vegetable Protease.—A commercial preparation of *papain* (*papayotin*, *carase* or *papase*), the protease of the fruit of the pawpaw (*carica papaya*), may be used in this connection. Follow the same procedure as that described under Gastric Protease (see p. 57).

It has been demonstrated by Mendel and Blood[5] that the presence of HCN will accelerate the proteolytic activity of papain. It is suggested that the HCN acts as a so-called *co-enzyme* (see page 47).

[1] If so desired, a solution of commercial pepsin powder in 0.2 per cent hydrochloric acid may be substituted for the extract of mucosa.

[2] Also called *trypsin*, *trypsase*, *pancreatic protease* and *alkali protease*. For a discussion of this enzyme see p. 256.

[3] A 0.25 per cent sodium carbonate solution of commercial *trypsin* or *pancreatin* may be substituted.

[4] A few c.c. of toluene or an alcoholic solution of thymol should be added to prevent putrefaction.

[5] Mendel and Blood: *Journal of Biological Chemistry*, 8, 177, 1910.

Vines[1] believes that "papain" consists of a mixture of two enzymes, a pepsin and an erepsin. Mendel and Blood do not consider the evidence on this point as conclusive.

III. LIPASES

1. Preparation of Pancreatic Lipase.[2]—An extract of this enzyme may be prepared from the pancreas of the pig or sheep according to the directions given on page 259.[3]

2. Demonstration of Pancreatic Lipase.—Into each of two test-tubes introduce 10 c.c. of milk and a small amount of litmus powder. To the contents of one tube add 3 c.c. of a neutral extract of pancreatic lipase and to the contents of the other tube add 3 c.c. of a boiled neutral extract of pancreatic lipase. Keep the tubes at 38°C. and watch for color changes.

The blue color of the litmus powder will gradually give place to a red. This change in color of the litmus from blue to red has been brought about by the fatty acid which has been produced through the lipolytic action exercised by the lipase upon the milk fats.

3. Preparation of Vegetable Lipase.—This enzyme may be readily prepared from castor beans, several months' old, by the following procedure.[4] Grind the shelled beans very fine[5] and extract for twenty-four-hour periods with alcohol-ether and ether, in turn. Reduce the semi-fat-free material to the finest possible consistency by means of mortar and pestle and pass this material through a sieve of very fine mesh. Place this material in a Soxhlet extractor and extract for one week. This fat-free powder may then be used to demonstrate the action of vegetable lipase. Powder prepared as described may be used in quantitative tests. For ordinary qualitative tests it is not necessary to remove the last traces of fat and therefore the extraction period in the Soxhlet apparatus may be much shortened.

4. Demonstration of Vegetable Lipase.—The lipolytic action of the lipase prepared from the castor bean, as just described, may be demonstrated in a manner entirely analogous to that used in the Demonstration of Pancreatic Lipase, see above. Proceed as indicated in that experiment and substitute the vegetable lipase powder for the neutral extract of pancreatic lipase. The type of action is entirely analogous in the two instances.

An experiment similar to Experiment 4, page 264, may also be tried if desired. In this experiment 0.2 c.c. of either *ethyl butyrate* or *amyl acetate* may be employed.

IV. INVERTASES[7]

1. Preparation of Vegetable Sucrase.[7]—Thoroughly grind about 100 grams of brewer's or baker's yeast in a mortar with sand. Spread the ground yeast in thin

[1] Vines: *Annals of Botany*, 19, 174, 1905.

[2] Also called steapsin. For a discussion of the enzyme see p. 258. A very active lipolytic extract may also be prepared from the liver.

[3] If preferred, a *glycerol extract* may be prepared according to the directions given by Kanitz (*Zeitschrift für physiologische Chemie*, 46, 482, 1906) or commercial *pancreatin* may be employed.

[4] A. E. Taylor: *On Fermentation; University of California Publications*, 1907.

[5] The shells should be removed without the use of water. These beans are *poisonous*, due to their content of *ricin*.

[6] *The inverting enzymes of the alimentary tract;* Mendel and Mitchell: *American Journa of Physiology*, 20, 81, 1907–08.

[7] For a discussion of this enzyme see p. 266.

layers on glass or porous plates and dry it rapidly in a current of dry, warm air. Powder this dry yeast, extract it with distilled water and filter. Pour the filtrate into acetone, stir and after permitting the acetone mixture to stand for a few minutes filter on a Buchner funnel. The resulting precipitate, after drying and pulverizing, may be used to demonstrate vegetable sucrase.

2. Demonstration of Vegetable Sucrase.—To about 5 c.c. of a 1 per cent solution of sucrose in a test-tube add a small amount of the sucrase powder prepared as directed above. Place the tube at 38°C. for 24–72 hours and at the end of that period test the solution by Benedict's test (see page 76.) Reduction indicates that the active sucrase powder has transformed the non-reducing sucrose into glucose and fructose, and these sugars, in turn, have reduced the Benedict solution.

For other experiments on Invertases, see Chapter XII.

V. OXIDASES[1]

1. Demonstration of Oxidase.—Oxidases or oxidizing enzymes constitute a very important group of intracellular enzymes. They are intimately connected with the oxidation processes in the plant and animal organisms.

1. Cut a thin slice from a freshly pared potato, place it on a watch glass and examine at intervals during the laboratory exercise. Note that the colorless potato gradually becomes brown.

This is due to the oxidation of para-oxyphenyl substances such as tyrosine, in the cells and in the intracellular juice of the potato. Two oxidases which have the power of accelerating the oxidation of para-oxyphenyl compounds are called *tyrosinase* and *laccase*.

2. Preparation of Potato Extract.—Scrape a pared potato by means of a knife or scalpel or comminute the potato substance by means of a grater. Extract the macerated potato substance by means of water. Strain through cheese cloth and filter the extract. Make an iodine test on the solid substance (see Starch, page 93), and save the water extract for use in the following experiments.

3. Oxidation of Para-oxyphenyl Compounds by Potato Oxidases.—Introduce 5 c.c. of filtered potato extract prepared as indicated above, into each of six test-tubes. Introduce additional reagents into the tubes according to the following series:

(a) Potato extract + 5 drops of toluene (control).
(b) Potato extract + 5 drops of ether (control).
(c) Potato extract + 5 drops of 1 per cent phenol solution.
(d) Potato extract + 5 drops of 1 per cent "tri-cresol" solution.
(e) Potato extract (boiled and cooled) + 5 drops of 1 per cent phenol solution.
(f) Potato extract (boiled and cooled) + 5 drops of 1 per cent "tri-cresol" solution.

Shake the contents of the six tubes thoroughly. Are there any immediate color changes? Place the tubes in your rack, and examine them at the next laboratory exercise.

[1] These experiments have been adapted from directions contained in the Laboratory Notes of Professor Gies of the College of Physicians and Surgeons, New York.

4. Experiments with Typical Oxidase Reagents.—Introduce 5 c.c. of filtered potato extract into each of four test-tubes. Add oxidase reagents as follows:

(a) Potato extract + 10 drops of guaiac solution.[1]

(b) Potato extract + 10 drops of α-naphthol solution.[2]

(c) Potato extract + 10 drops of para-phenylenediamine hydrochloride solution.[3]

(d) Potato extract + 5 drops of α-naphthol solution + 5 drops of para-phenylenediamine hydrochloride solution + 5 drops of 10 per cent sodium carbonate (Indophenol Test).

Shake the contents of each tube thoroughly and note immediate color changes. Place the tubes in the rack and leave them undisturbed until the end of the laboratory exercise. Note any changes or peculiarities in the coloration effects, especially at the surface of the liquid.

In tube (a) the guaiaconic acid of the guaiac resin has been oxidized and formed *guaiac blue.*

In tube (b) a *violet* coloration due to the production of di-naphthol appears. The oxidase has oxidized the α-naphthol.

In tube (c) we have a change whose chemistry is not well known.

In tube (d) we have the production of indophenol from the α-naphthol and the para-phenylenediamine hydrochloride under the influence of oxidase. The indophenol is soluble in the alkaline solution. The color gradually changes from *red* to *purple* as the indophenol accumulates.

The production of the above colors does not possess any biological significance. These colors simply serve to indicate that certain reactions are taking place which occur normally in living cells, although in the latter case they are of course unaccompanied by any color change. Intracellular oxidase favors the utilization of oxygen by a cell, just as the potato oxidase has facilitated the oxidation of the chromogens in the above tests.

5. Experiments Using Hydrogen Peroxide.—Into each of four test-tubes place 5 c.c. of filtered potato extract prepared as in Experiment 2, page 59. Prepare a second series of four tubes (see 4, above), but use a boiled potato extract. Prepare also a third series using water instead of potato extract. Now add to each of the twelve tubes 5 drops of a 3 per cent solution of hydrogen peroxide. While the resultant lively effervescence is in progress add to each series the four "Typical Oxidase Reagents" in the order and quantities specified in the preceding experiment (4). Allow the tubes to remain undisturbed and carefully note comparative effects during the remainder of the laboratory exercise.[4] Compare with results of experiment (4).

6. The Schardinger Reaction.—Place 5 c.c. of milk in each of three test tubes. Heat No. 1 to boiling and cool. Add to each tube 1 c.c. of 0.02 per cent methylene

[1] Made by dissolving 0.5 gram of guaiac resin in 30 c.c. of 95 per cent alcohol.
[2] Made by dissolving 1 gram of α-naphthol in 100 c.c. of 95 per cent alcohol.
[3] Dissolve 1 gram of para-phenylenediamine hydrochloride in 100 c.c. of water.
[4] This experiment has been adapted from one contained in the Laboratory Notes of Professor Gies of the College of Physicians and Surgeons, New York.

blue solution. To Nos. 1 and 2 add 1 c.c. of 0.4 per cent formaldehyde solution. Mix by gentle rotation—add 1–2 c.c. of paraffin oil and put in a water bath about 40°C. The milk in tube 2 should gradually decolorize. The reaction is an example of anaerobic oxidation in the presence of a hydrogen acceptor (methylene blue) and may be written thus:

$$CH_2O + H_2O \rightarrow CH_2(OH)_2 + MB \rightarrow HCOOH + MB{\cdot}H_2.$$

This reaction is given more slowly by milk which has been heated and more rapidly by milk which has a high bacterial count.

7. Study of Tissue Oxidations by the Methylene Blue Method of Thunberg and Ahlgren.[1] Principle.—Finely divided tissue is suspended in a solution containing methylene blue, phosphate solution to regulate the acidity, and the substance whose action it is desired to determine. The tube is evacuated placed in a water bath and the time required for the methylene blue to be decolorized is determined. This is a measure of the rate of oxidation in the mixture. The nature of the substances capable of being oxidized by the tissue can be determined and the influences governing the oxidation process studied.

Procedure.—The tube[2] most convenient for use in this experiment is illustrated in Fig. 14. Into each of three tubes introduce 0.9 c.c. of a mixture of 8 c.c. of methylene blue 1:2000 and 6 c.c. M/10 K_2HPO_4. Then add 0.1 c.c. of water to the first tube, of M/10 potassium succinate to the second and of M/10 potassium glycerophosphate to the third. Then add 0.2 gm. of finely divided washed rabbit muscle[3] to each. Evacuate each tube for two or three minutes with a strong water jet filter pump.[4] Place the tubes in a constant temperature water bath at 35°. Observe from time to time and note when each tube just loses its last trace of blue color. Record the number of minutes required in each case. Do the succinate and glycerophosphate solutions decolorize first? What does

[1] Ahlgren: *Skand. Arch. f. Physiol.*, Supplement to Vol. 47, 1925.

[2] The tube is made of hard, colorless glass and is of about 10 c.c. capacity. The stopper is greased with a mixture of: rubber 1 part, petroleum jelly 2 parts, and heavy liquid petrolatum 1 part. Tubes may be obtained from Rudolph Grave, Malmskillnadsgatan 48 C, Stockholm.

Solutions required are: Stock solution of methylene blue (Merck Medicinal) one gram in 500 c.c. of water. Phosphate buffer solution: An M/10 solution of K_2HPO_4 made by dissolving 17.33 gm. in 1000 c.c. of water.

[3] The muscles of frogs and other animals may also be used. The animals should be killed by a blow and not by anaesthesia. One or more gm. of muscle tissue freed from ligaments, fat and blood as much as possible are cut up on a watch glass with a scissors of the Cooper type for three to five minutes. A drop or two of water may be added to assist cutting. The tissue may then be washed if desired by shaking with an excess of water, rubbing in a mortar, straining through silk and drying with filter paper. Keep in a covered beaker placed in a dish containing pieces of ice. The divided tissue is best used as soon as possible but may generally be kept for an hour at 20°. Uncut tissue may be kept somewhat longer. In weighing the tissue a microbalance of the Hartmann and Braun or other type weighing up to 500 mg. is convenient, a small celluloid shell being attached to hold the material. A glass rod with a spoon shaped tip is used to introduce the tissue into the tube.

[4] There may be some foaming during the evacuation which should be continued until the solution boils when warmed with the hand. Foaming may be reduced by rotating the tube in a horizontal position so as to distribute the material on the sides of the tube or by first putting the tubes in cold water. After evacuation to get all material into the bottom of the tubes they may be rotated for a few seconds in a hand centrifuge. It is also well after evacuation and closing the stopper to remove the tube from the side tube while holding both under water. The side tube is filled with water and leakage of air is less probable.

this indicate as to the oxidation of these substances by muscle tissue? Glucose is not oxidized under these conditions but there is some evidence that it may be oxidized if insulin is added.

8. Nitroprusside Reaction for Glutathione.—Rub up in a mortar a small piece of fresh liver or kidney tissue, a little sand and a few c.c. of saturated ammonium sulphate solution. Add 5 or 6 drops of 2 per cent sodium nitroprusside and

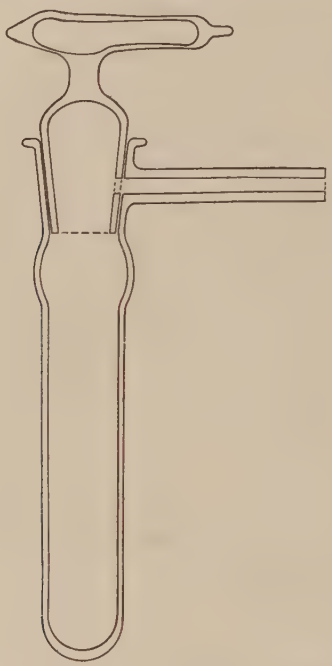


FIG. 14.—VACUUM TUBE. (*Thunberg*).

2 c.c.of concentrated ammonium hydroxide solution. The mixture turns purple.

9. Estimation of Glutathione in Tissue.[1]—A trichloracetic acid extract is titrated with iodine. The reaction is:

$$2GSH + I_2 \rightarrow GS - SG + 2HI.$$

Procedure.—Grind a known weight of tissue in a mortar with sand and 10 per cent trichloracetic acid. Filter and re-extract the residue twice with acid. Titrate with 0.01 N iodine solution using sodium nitroprusside as an external indicator (glutathione gives a color reaction with nitroprusside). One c.c. of 0.01 N iodine is equivalent to 2.5 mg. reduced glutathione. Glutathione is absent from whole blood. Skeletal muscle of the rat contains 0.034 per cent, liver 0.18 per cent, yeast 0.18 per cent.

VI. CATALASE

Demonstration of Catalase.—The various animal tissues as liver, kidney, blood, lung, muscle and brain contain enzymes called catalases

[1] Tunnicliffe: *Biochem. J.*, 19, 194, 1925.

which possess the property of decomposing hydrogen peroxide with the production of molecular oxygen. Catalase is also found in many plant tissues.

1. Animal Catalase.—The presence of this enzyme may also be demonstrated as follows: Introduce into a low, broad, wide-mouthed bottle some pulped liver tissue and a porcelain crucible containing *neutral* hydrogen peroxide.[1] Connect the bottle with a eudiometer filled with water, upset the crucible of hydrogen peroxide upon the liver pulp and note the collection of gas in the eudiometer. This gas is oxygen which has been liberated from the hydrogen peroxide through the action of the catalase of the liver tissue.

See next experiment for a method for the quantitative determination of catalase based on the above principle.

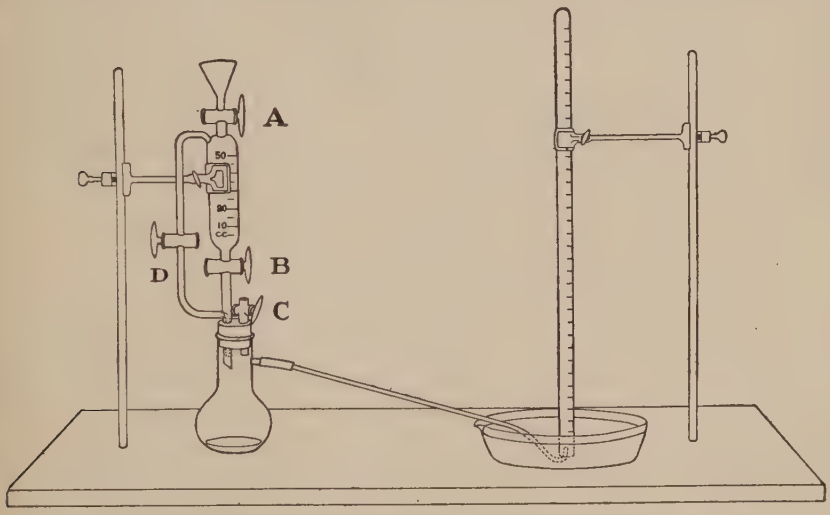


FIG. 15.—APPARATUS FOR QUANTITATIVE DETERMINATION OF CATALASE.

2. Quantitative Determination of Catalase.[2]—In the determination of the catalase values of tissues weighed portions of the tissue under examination should be ground with sand in a mortar then treated with four volumes of chloroform water and permitted to extract for 24 hours at room temperature. An apparatus such as that shown in Fig. 15 may be employed in determining the catalase values.[3] The main features of the apparatus are based upon those of a delivery funnel for introducing liquids under increased or diminished pressure.

In making a determination introduce a measured volume (1–4 c.c.) of the filtered extract[4] into the small flask and insert the modified Johnson burette graduated to 5 c.c. and containing 50 c.c. of hydrogen peroxide (Oakland dioxygen, neutral[5] to Congo red) into the neck of the flask. Fill the eudiometer with water and place in

[1] Mendel and Leavenworth: *American Journal of Physiology*, 21, 85, 1908.

[2] Hawk: *Journal of the American Chemical Society*, 33, 425, 1911.

[3] Another type of apparatus has been suggested by Burge (*Am. Jour. Physiol.* 41, 153, 1916).

[4] If less than 4 c.c. of extract are used the volume should be made up to 4 c.c. by the addition of distilled water.

[5] An acid reaction modifies the rate of the oxygen evolution. (See Mendel and Leavenworth, *American Journal of Physiology*, 21, 85, 1908.)

position. Close cocks A and C and open cocks B and D thus permitting 5 c c. of the peroxide to flow into the flask. Shake the contents of the flask briskly[1] and record the volume of oxygen evolved in a two-minute period taking readings at intervals of fifteen seconds.

Calculation.—When a series of comparative tests are made on different tissues or on the same tissue under different conditions it is considered satisfactory to make a comparison of the catalase values upon the basis of the *volume of oxygen evolved in a period of two minutes from 5 c.c. of neutral hydrogen peroxide by means of 1 c.c. of a 1 : 4 chloroform-water extract of the tissue.*

VII. UREASE

Preparation of Crystalline Urease.—Dilute 158 c.c. of redistilled acetone[2] to 500 c.c. of 22°C. (= 31.6 per cent acetone). Pour over 100 gm. Arlco jack bean meal in a beaker. Stir 3 to 4 minutes and filter through a Schleicher and Schüll, No. 595, filter. Return the first filtrate until clear. Complete filtration in ice chest at 2–2.5°C., overnight. Centrifuge off the crystals that form using cold centrifuge tubes. Examine microscopically (see Fig. 12A). Drain and stir up with 5 to 10 c.c. of cold 31.6 per cent acetone. Centrifuge again. Dissolve crystals in 15–40 c.c. distilled water at room temperature and free the solution from insoluble matter by centrifugation. The activity of the concentrated solution is not lost very rapidly provided the material is kept in the ice chest.

C. Experiments on Anti-Enzymes

1. Preparation of an Extract of Anti-Pepsin.[3]—Grind up a number of intestinal worms (ascaris)[4] with quartz sand in a mortar. Subject this mass to high pressure, filter the resultant juice and treat it with alcohol until a concentration of 60 per cent is reached. If any precipitate forms it should be filtered off[5] and alcohol added to the filtrate until the concentration of alcohol is 85 per cent, or over. The anti-enzyme is precipitated by this concentration. Permit this precipitate to stand for twenty-four hours, then filter it off, wash it with 95 per cent alcohol, absolute alcohol, and ether, in turn, and finally dry the substance over sulphuric acid. The sticky powder which results may be used in this form or may be dissolved in water as desired and the aqueous solution used.[6]

2. Demonstration of Anti-Pepsin.[7]—Introduce into a test-tube a few fibrin shreds and equal volumes of pepsin-hydrochloric acid[8] and ascaris extract made as indicated above. Prepare a control tube in which the ascaris extract is replaced by water. Place the tubes at 38°C. Ordinarily in one hour the fibrin in the control tube will be completely digested. The fibrin in the tube containing the ascaris

[1] In making a series of comparative tests it is essential that the shaking process should be uniform from determination to determination.

[2] Commercial acetone distilled over fused $CaCl_2$ and a little soda-lime, to remove water and acid.

[3] Anti-gastric-protease or anti-acid-protease.

[4] These may be readily obtained from pigs at a slaughter house.

[5] This precipitate consists of impurities, the anti-enzyme not being precipitated until a higher concentration of alcohol is reached.

[6] The original ascaris extract possesses much greater activity than either the powder or the aqueous solution.

[7] Martin H. Fischer: *Physiology of Alimentation*, 1907, p. 134.

[8] Made by bringing 0.015 gram of pepsin into solution in 7 c.c. of water and 0.23 gram of concentrated hydrochloric acid

extract may, however, remain unchanged for days, thus indicating the inhibitory influence exerted by the anti-enzyme present in this extract.

3. Preparation of an Extract of Anti-Trypsin.[1]—The extract may be prepared from the intestinal worm, ascaris, according to the directions given above.

4. Demonstration of Anti-Trypsin.—Introduce into a test-tube a few shreds of fibrin and equal volumes of an artificial tryptic solution[2] and the ascaris extract made as described on page 64. Prepare a control tube in which the ascaris extract is replaced by water. Place the two tubes at 38°C.

Ordinarily the fibrin in the control tube will be completely digested in two hours. The fibrin in the tube containing the ascaris extract may, however, remain unchanged for days, thus indicating the inhibitory influence of the anti-enzyme.

Blood serum also contains *anti-trypsin*. This may be demonstrated as follows: Introduce equal volumes of serum and artificial tryptic solution (prepared as described above) into a test-tube and add a few shreds of fibrin. Prepare a *control* tube containing *boiled* serum. Place in two tubes at 38°C. It will be observed that the fibrin in the tube containing the *boiled* serum digests, whereas that in the other tube does not digest. The anti-trypsin present in the unboiled serum has exerted an inhibitory influence upon the action of the trypsin.

D. Quantitative Applications

Methods for the quantitative determination of various enzymes will be found in the following chapters: **Amylase** (Chapter XI); **Erepsin** (Chapter XII); **Pepsin** (Chapter IX); **Trypsin** (Chapter XI). For the application of **Urease** to the determination of urea, see Chapters XVIII and XXXI.

[1] Anti-pancreatic-protease or anti-alkali-protease.

[2] Made by dissolving 0.04 gram of sodium carbonate and 0.015 gram of trypsin in 8 c.c. of water.

CHAPTER III

CARBOHYDRATES

THE name carbohydrates is given to a class of bodies which are an especially prominent constituent of plants and which are found also in the animal body either free or as an integral part of various proteins. They are called carbohydrates because they contain the elements C, H and O; the H and O being present in the proportion to form water. The term is not strictly appropriate inasmuch as there are bodies, such as acetic acid, lactic acid and inositol, which have H and O present in the proportion to form water, but which are not carbohydrates, and there are also true carbohydrates which do not have H and O present in this proportion, *e.g.*, rhamnose, $C_6H_{12}O_5$.

Chemically considered, the carbohydrates are aldehyde or ketone derivatives of complex alcohols. Treated from this standpoint, the aldehyde derivatives are spoken of as aldoses, and the ketone derivatives are spoken of as ketoses. The carbohydrates are also frequently named according to the number of oxygen atoms present in the molecule, *e.g.*, trioses, pentoses, and hexoses.

The carbohydrates are also frequently classified according to the number of simple carbohydrate groups which they contain as mono-, di-, tri- and polysaccharides. The monosaccharides contain only a single such group and hence can not be hydrolyzed to form simpler sugars. To this class belong the hexoses (general formula $C_6H_{12}O_6$) and the pentoses (general formula $C_5H_{10}O_5$). The disaccharides give two molecules of simple sugars on hydrolysis and the more common ones have the general formula $C_{12}H_{22}O_{11}$. The polysaccharides give several molecules of simple sugars on hydrolysis and the formula for the more important members of this group is $(C_6H_{10}O_5)_x$ where x represents the number of simple sugar groups present. In a general way the solubility of the carbohydrates varies with the complexity, the more complex being the least soluble. This means simply that, as a class, the monosaccharides (hexoses) are the most soluble and the polysaccharides (starches and cellulose) are the least soluble. The name *glucide* has been suggested as a group name for the carbohydrates and glucosides.

The more common carbohydrates may be classified as follows:

I. Monosaccharides.
  1. Pentoses, $C_5H_{10}O_5$.
    (*a*) Arabinose.
    (*b*) Xylose.
    (*c*) Rhamnose (Methyl-pentose), $C_6H_{12}O_5$.
  2. Hexoses, $C_6H_{12}O_6$.
    (*a*) Glucose.
    (*b*) Fructose.
    (*c*) Galactose.

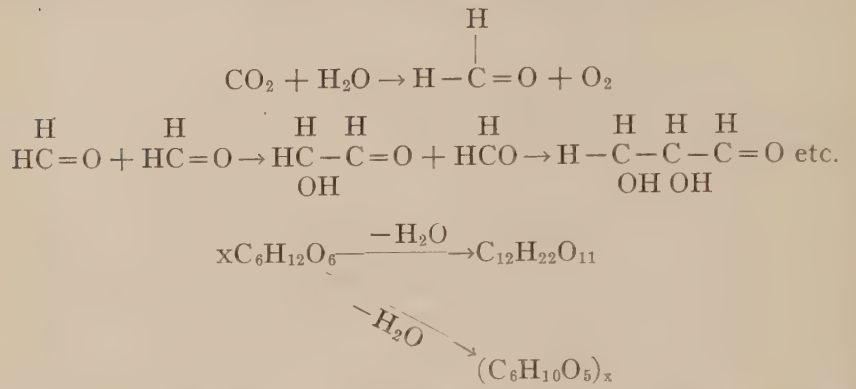
II. Disaccharides, $C_{12}H_{22}O_{11}$.
  1. Maltose.
  2. Lactose.
  3. Iso-Maltose.
  4. Sucrose.

III. Trisaccharides, $C_{18}H_{32}O_{16}$.
  1. Raffinose.

IV. Polysaccharides, $(C_6H_{10}O_5)_x$.
  1. Gum and Vegetable Mucilage Group.
    (*a*) Dextrin.
    (*b*) Vegetable Gums.
  2. Starch Group.
    (*a*) Starch.
    (*b*) Inulin.
    (*c*) Glycogen.
    (*d*) Lichenin.
  3. Cellulose Group.
    (*a*) Cellulose.
    (*b*) Hemicelluloses.
      (1) Pentosans.
        Gum Arabic.
      (2) Hexosans.
        Galactans.
          Agar-agar.

Photosynthesis.—It is interesting to note that these highly complex substances whose structures, especially the polysaccharides, are as yet unknown are formed by plants from water and such simple compounds as the carbon dioxide absorbed by the leaves. Although the mechanism of these reactions is still obscure, it has long been recognized that these syntheses are related to sunlight and to the presence of chlorophyll, or

other such coloring matter, in the plant. Baeyer suggested that formaldehyde was formed as an intermediate product which polymerized to form the monosaccharides. These sugars could then condense to form the more complex di- and polysaccharides, as indicated below.



Willstätter pointed out, further, that formaldehyde is the only possible intermediate product which is capable of giving the experimentally observed "assimilatory quotient" of 1, that is, of giving one volume of oxygen for each volume of carbon dioxide absorbed.

During the last decade, a great deal of experimental work has been done in an attempt to determine the exact nature of the intermediate product, if any, formed in carbohydrate synthesis and the functions of both the chlorophyll and the sunlight involved in the reaction. Although the problem is still unsolved, the experiments of Baly and others with water solutions of carbon dioxide exposed under various conditions to ultraviolet light (Chap. XXXII) from a quartz mercury lamp, indicate that formaldehyde is formed when the solutions are exposed to rays having a wave length of 200$\mu\mu$. Further, if rays having a wave length of 290$\mu\mu$ are present at the same time, the formaldehyde formed is immediately polymerized into reducing sugars. This latter result explains the earlier observation, which was used to combat the Baeyer hypothesis, that plant leaves contain no measureable amount of formaldehyde. Baly also concludes that chlorophyll acts as a photocatalyst, or light screen, absorbing rays of light having wave lengths of 350$\mu\mu$ or longer and then radiating them at frequencies capable of accomplishing the various steps in the carbohydrate synthesis. The active agent in the synthesis of formaldehyde is really the ultraviolet waves of short wave length, but since ordinary sunlight, after passing through the atmosphere, is comparatively poor in these rays, the chlorophyll seems to make possible the utilization by the plant of the more abundant rays of longer wave lengths.

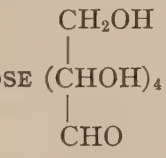
Plants also absorb soluble nitrates, or nitrites, and possibly even nitrogen from the air. These, together with the formaldehyde formed photosynthetically, are utilized by the plant in its synthesis of such complex nitrogen derivatives as the alkaloids and proteins.

MONOSACCHARIDES

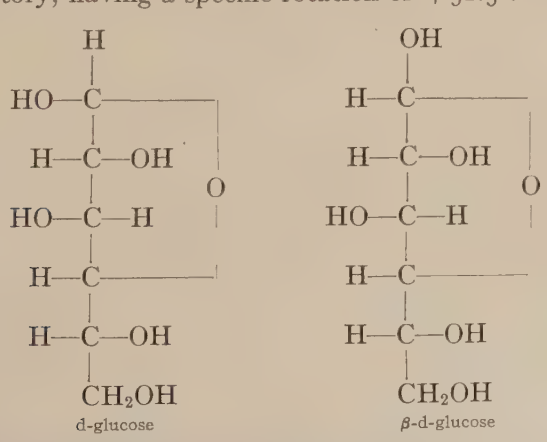
Hexoses, $C_6H_{12}O_6$

The hexoses are monosaccharides containing six oxygen atoms in a molecule. They are the most important of the simple sugars, and two of the principal hexoses, glucose and fructose, occur widely distributed in plants and fruits. Of these two hexoses, glucose results from the hydrolysis of starch, whereas both glucose and fructose are formed in the hydrolysis of sucrose. Galactose, which with glucose results from the hydrolysis of lactose, is also an important hexose. These three hexoses are fermentable by yeast, and yield levulinic acid upon heating with dilute mineral acids. They reduce metallic oxides in alkaline solution, are optically active and extremely soluble. With phenylhydrazine they form characteristic osazones.

GLUCOSE



Glucose, also called dextrose or grape sugar, is present in the blood in small amount and may also occur in traces in normal urine.[1] It occurs, together with fructose, in the juice of many fruits, and is obtained commercially by the hydrolysis of starch. In stable solutions, it is dextrorotatory, having a specific rotation of $+52.5°$.



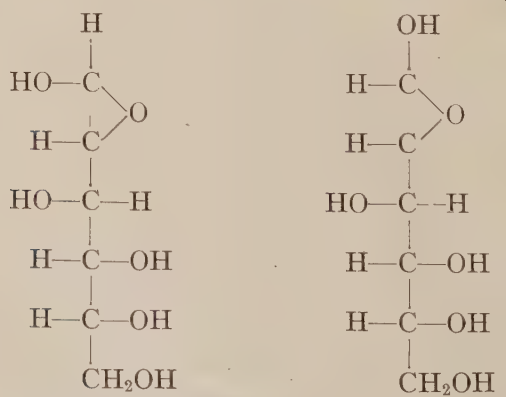
d-glucose β-d-glucose

[1] See Folin's test for sugar in normal urine (*Jour. Biol. Chem.*, 22, 327, 1915): Benedict: *Jour. Biol. Chem.*, 31, 195, 1918: Sumner: *Jour. Biol. Chem.*, 62, 287, 1924, and Benedict: *Jour. Biol. Chem.*, 68, 759, 1926.

The behavior of glucose solutions indicates that only a small fraction of the substance is present in the free aldehyde form, the remainder existing in a less active modification in which the carbon atom of the aldehyde group is linked to the fourth carbon atom by means of an atom of oxygen. (See formulae.) This type of linkage is known as the butylene oxide ring and since, in its formation, the first carbon atom has become asymmetric, we find that two modifications of this form exist: the first, known as α-d-glucose, has a specific rotation of $+113.4^{\circ}$ and the second, known as β-d-glucose, has a specific rotation of $+19^{\circ}$. In solution, these forms are in equilibrium with each other and probably also with a small amount of the more active form having the free aldehyde group, the specific rotation of the equilibrium mixture, which is the rotation ordinarily measured, being $+52.5^{\circ}$.

In freshly prepared solutions of glucose, the α form exists alone, the solution having a specific rotation of $+113.4^{\circ}$. On standing, however, some of the molecules are slowly transformed into the β form and an equilibrium is finally established between the two forms. A trace of alkali hastens the formation of this equilibrium. The specific rotation of the solution falls as the amount of the β form increases until equilibrium, with its characteristic specific rotation of $+52.5^{\circ}$, is reached. This phenomenon is known as mutarotation.

In addition to these butylene oxide configurations, glucose may exist in forms where the terminal carbon atom is linked, through an oxygen atom, to other carbon atoms than the fourth thus forming so-called ethylene, propylene or amylene oxide rings, each of which may also exist in the α and β forms. These modifications of glucose are all grouped together under the name γ glucose, and are supposed to be much more reactive than the ordinary forms.



α and β forms respectively, of γ glucose (ethylene oxide structure)

γ glucose has a lower optical activity, however, and it is to this fact that discrepancies between copper reduction and optical rotation methods, have been attributed. There is reason to believe that the normal organism converts ordinary glucose to the γ variety as a preliminary to oxidation, a power which is lacking in diabetics.[1]

Glucose is one of the sweetest of the common sugars, being excelled only by fructose and sucrose.[2] The following table gives the relative sweetness of some of the sugars considering sucrose as 100:

Sugar	Relative sweetness sucrose = 100
Lactose	16.0
Raffinose	22.6
Galactose	32.1
Rhamnose	32.5
Maltose	32.5
Xylose	40.0
Glucose	74.3
Sucrose	100.0
Fructose	173.3

Invert sugar is about 30 per cent sweeter than sucrose.[3]

Experiments on Glucose

The following tests are made on glucose as a typical carbohydrate and are *not specific for this sugar.* A specific test for glucose is the Phenylhydrazine Reaction (3) in the absence of a positive Resorcinol-Hydrochloric Acid Reaction (see page 84).

1. Solubility.—Test the solubility of glucose in the "ordinary solvents" and n alcohol. (In the solubility test throughout the book we shall designate the following solvents as the "ordinary solvents;" H_2O; 10 per cent NaCl; 0.5 per cent Na_2CO_3; 0.2 per cent HCl; concentrated KOH; concentrated HCl.)

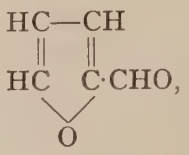
2. α-Naphthol Reaction (Molisch).—Place approximately 5 c.c. of concentrated H_2SO_4 in a test-tube. Incline the tube and slowly pour down the inner side of it approximately 5 c.c. of the sugar solution to which 2 drops of Molisch's reagent (a 15 per cent alcoholic solution of α-naphthol) has been added, so that the sugar solution will not mix with the acid. A reddish-violet zone is produced at the point of contact.

[1] For a review of the "Intermediary Metabolism of Carbohydrates," see Shaffer, *Physiol. Rev.*, 3, 394, 1923.

[2] Biester, Wood & Wahlin: *Am. Jour. Physiol.*, 73, 387, 1925.

[3] Willaman, Wahlin & Biester: *Am. Jour. Physiol.*, 73, 397, 1925.

The reaction is due to the formation of furfural,[1]



by the acid. The test is given by all bodies containing a carbohydrate group and is therefore not specific and, in consequence, of very little practical importance.

3. Phenylhydrazine Reaction.—Test according to one of the following methods: (a) To a small amount of phenylhydrazine mixture (enough to fill the rounded portion of a small test-tube) furnished by the instructor,[2] add 5 c.c. of the sugar solution, shake well and heat on a boiling water-bath for one-half to three-quarters of an hour. Allow the tube to cool slowly (not under the tap) and examine the crystals microscopically (Plate III, opposite).

If the solution has become too concentrated in the boiling process it will be light red in color and no crystals will separate until it is diluted with water.

Yellow crystalline bodies called *osazones* are formed from certain sugars under these conditions, in general each individual sugar giving rise to an osazone of a definite crystalline form which is typical for that sugar. It is important to remember in this connection that, of the simple sugars of interest in physiological chemistry, glucose and fructose yield the same osazone. The melting points of the recrystallized osazones may be used as a further means of identification but since they extend over a range of several degrees and are not far apart for the different sugars, the determination of melting points is of doubtful value. Garard and Sherman[3] claim that "With the best conditions it is possible to detect 5 mg. of glucose in 10 cc. of solution since a distinct precipitate is formed. If the solution is cooled, 1 mg. gives a distinct precipitate. If the original solution is neutral and comparatively free from other organic matter this test will show 1 part of glucose in 10,000 of water or 1 mg. in a 0.01 per cent. solution. At this dilution the precipitate is sufficiently copious, so that there is no question about its presence."

[1] According to v. Ekenstein and Blanksma (*Ber. d. d. chem. Gesell.*, 43, 2358, 1910) oxymethylfurfural is formed.

[2] This mixture is prepared by combining 2 parts of phenylhydrazine hydrochloride and 3 parts of sodium acetate *by weight*. These are thoroughly mixed in a mortar.

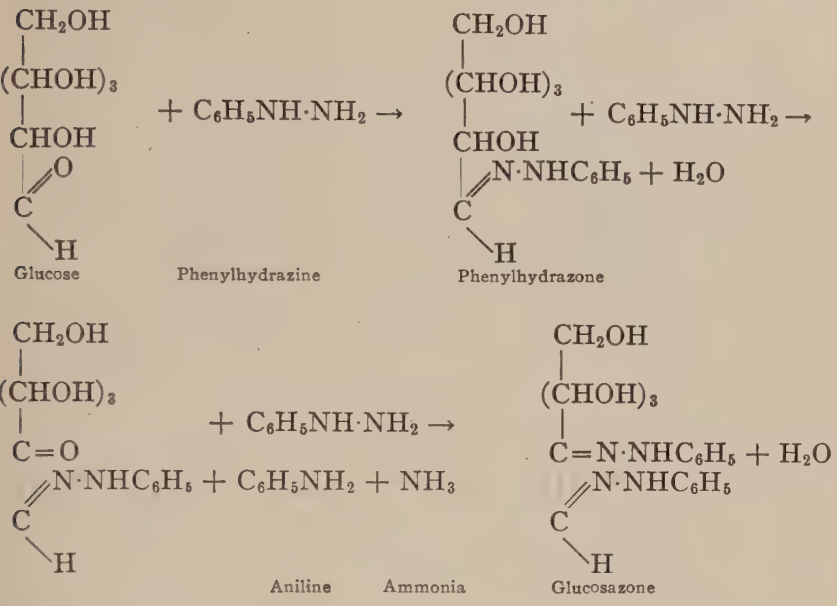
[3] Garard and Sherman: *Jour. Am. Chem. Soc.*, 40, 955, 1918.

PLATE III.

OSAZONES.

Upper form, dextrosazone; central form, maltosazone; lower form, lactosazone.

The reaction taking place in the formation of *phenylglucosazone* is as follows:



(b) Place 5 c.c. of the sugar solution in a test-tube, add 1 c.c. of the phenylhydrazine-acetate solution furnished by the instructor,[1] and heat on a boiling water-bath for one-half to three-quarters of an hour. Allow the liquid to cool slowly and examine the crystals microscopically (Plate III, opposite p. 72).

4. Diffusibility of Glucose.—Test the diffusibility of glucose solution through animal membrane or parchment paper, making a dialyzer like one of the models shown in Fig. 16.

A most satisfactory dialyzing bag may be made of collodion as follows: Pour a solution of collodion into a clean dry Erlenmeyer flask or test-tube. While rotating the vessel on its longitudinal axis, gradually pour out the collodion, at the same time being careful that the interior surface of the flask is completely coated with the solution. Continue the rotation in the inverted position until the collodion ceases to flow. After the solution has evaporated such that the collodion skin on the rim is dry and stiff, cut or loosen it around the edge of the rim. With a pipette or wash bottle run in a few cubic centimeters of water between the membrane and the wall of the flask or test-tube. Shake the inclined vessel while rotating on its longitudinal axis, thus detaching the membrane. Now withdraw the detached bag and fill with water, to determine whether or not it contains defects.[2]

All monosaccharides and disaccharides are diffusible, but many polysaccharides are not.

[1] This solution is prepared by mixing one part *by volume*, in each case, of glacial acetic acid, one part of water and two parts of phenylhydrazine (the base).

[2] Gies: Quoted by Clark. *Bioch. Bull.*, 1, 198, 1911.

A continuous dialysis or extraction apparatus operating at reduced pressure with a constant volume of fluid has been described by Hanke and Koessler.[1]

5. **Influence of Alkali (Moore's Test).**—To 2–3 c.c. of sugar solution in a test-tube add an equal volume of concentrated KOH or NaOH, and boil. The solution

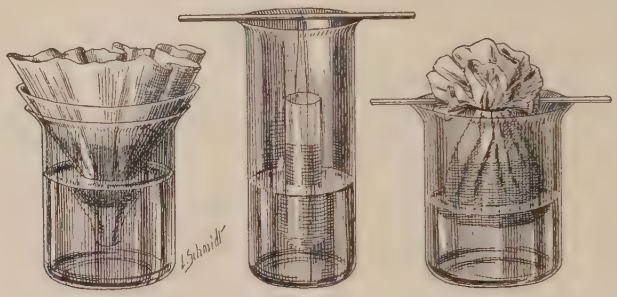
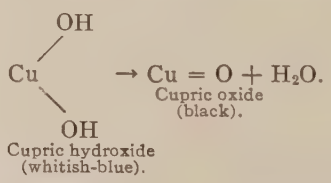


FIG. 16.—DIALYZING APPARATUS FOR STUDENTS' USE.

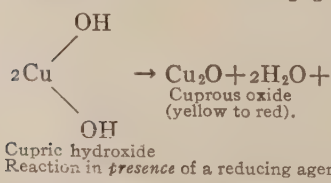
darkens and finally assumes a brown color. At this point the odor of caramel may be detected.

This test is of little practical value for the detection of glucose. The alkali brings about condensation and decomposition. The brown color is due to the formation of condensation products. Among the decomposition products are the potassium or sodium salts of certain organic acids.

6. Reduction Tests.—To their aldehyde or ketone structure many sugars owe the property of readily reducing alkaline solutions of the oxides of metals like copper, bismuth and mercury; they also possess the property of reducing ammoniacal silver solutions with the separation of metallic silver. Upon this property of reduction the most widely used tests for sugars are based. When whitish-blue cupric hydroxide in suspension in an alkaline liquid is heated it is converted into insoluble black cupric oxide, but if a reducing agent like certain sugars be present the cupric hydroxide is reduced to insoluble yellow or red cuprous oxide. These changes are indicated as follows:



Reaction in *absence* of a reducing agent.



Reaction in *presence* of a reducing agent.

[1] Hanke & Koessler: *Jour. Biol. Chem.*, 166, 495, 1925.

The chemical equations here discussed are exemplified in Fehling's test.

(*a*) *Trommer's Test.*—To 5 c.c. of sugar solution in a test-tube add one-half its volume of KOH or NaOH. Mix thoroughly and add, drop by drop, a *very dilute* solution of copper sulphate. Continue the addition until there is a slight permanent precipitate of cupric hydroxide and in consequence the solution is slightly turbid. Heat, and the cupric hydroxide is reduced to yellow or brownish-red cuprous oxide.

If the solution of copper sulphate used is too strong a small brownish-red precipitate produced in a weak sugar solution may be entirely masked. On the other hand, particularly in testing for sugar in the urine, if too little copper sulphate is used a light-colored precipitate formed by uric acid and purine bases may obscure the brownish-red precipitate of cuprous oxide. The action of KOH or NaOH in the presence of an excess of sugar and insufficient copper will produce a brownish color. Phosphates of the alkaline earths may also be precipitated in the alkaline solution and be mistaken for cuprous oxide. Trommer's test is not very satisfactory.

Salkowski[1] has proposed a modification of the Trommer procedure which he claims is a very accurate sugar test.

(b) **Fehling's Test.—To about 1 c.c. of Fehling's solution[2] in a test-tube add about 4 c.c. of water, and boil.[3] [The cupric hydroxide is held in solution by the sodium potassium tartrate (Rochelle salt).] This is done to determine whether the solution will of itself cause the formation of a precipitate of brownish-red cuprous oxide. If such a precipitate forms, the Fehling's solution must not be used. Add sugar solution to the warm Fehling's solution a few drops at a time and heat the mixture after each addition. The production of yellow or brownish-red cuprous oxide indicates that reduction has taken place. The yellowish precipitate is more likely to occur if the sugar solution is added rapidly and in large amount, whereas with a less rapid addition of smaller amounts of sugar solution the brownish-red precipitate is generally formed. The differences in color of the cuprous oxide precipitates under different conditions are apparently due to differences in the size of the particles, the more finely divided precipitates having a yellow color, while the coarser ones are red. In the presence of protective colloidal substances the yellow precipitate is usually formed.**[4]

In testing a solution preserved by *chloroform* a positive test may be obtained in the absence of sugar. This is due to the fact that the hot alkali produces formic acid (a reducing fatty acid) from the chloroform.

[1] Salkowski: *Zeit. physiol. Chem.*, 79, 164, 1912.

[2] Fehling's solution is composed of two definite solutions—a copper sulphate solution and an alkaline tartrate solution—which may be prepared as follows:

Copper sulphate solution = 34.65 grams of copper sulphate dissolved in water and made up to 500 c.c.

Alkaline tartrate solution = 125 grams of potassium hydroxide and 173 grams of Rochelle salt dissolved in water and made up to 500 c.c.

These solutions should be preserved separately in rubber-stoppered bottles and mixed in equal volumes when needed for use. This is done to prevent deterioration.

[3] More dilute Fehling's solution should be used in testing very dilute sugar solutions. In case of concentrated sugar solutions it may sometimes be desirable to use a larger volume of the Fehling's solution.

[4] Fischer and Hooker: *Science*, N. S., 45, 505, 1917.

Ammonium salts also interfere with Fehling's test. If present in excess the solution (*e.g.*, urine) should be made alkaline and boiled in order to decompose the ammonium salts.

If the solution under examination by Fehling's test is acid in reaction it must be neutralized or made alkaline before applying the test.

(c) Benedict's Test.[1]—Benedict has modified the Fehling solution and has succeeded in obtaining one which does not deteriorate upon long standing.[2] The following is the procedure for the detection of glucose in solution. To 5 c.c. of the reagent in a test-tube add 8 (not more) drops of the solution under examination. Boil the mixture vigorously for two minutes and then allow the fluid to cool spontaneously (Do not hasten cooling by immersion in cold water). In the presence of dextrose the entire body of the solution will be filled with a colloidal precipitate, which may be red, yellow or green in color, depending upon the amount of sugar present. In the presence of over 0.2–0.3 per cent of glucose the precipitate will form quickly. If no glucose is present, the solution will remain perfectly clear. (If urine is being tested, it may show a very faint turbidity, due to precipitated urates.)

Even very small quantities of glucose (0.1 per cent) yield precipitates of surprising bulk with this reagent, and the positive reaction for glucose is the filling of the entire body of the solution with a precipitate, so that the solution becomes opaque. Since *amount* rather than color of the precipitate is made the basis of this test, it may be applied even for the detection of small quantities of glucose, as readily in artificial light as in daylight. Chloroform does not interfere with this test nor do uric acid or creatinine interfere to such an extent as in the case of Fehling's test.

(d) Folin-McEllroy Test.[3]—To 5 c.c. of the reagent[4] in a test tube add 5–8 drops of the solution under examination (never add more than 0.5 c.c.) and boil for 1–2 minutes or heat in a beaker of boiling water for 3 minutes. If more than the normal traces of sugar be present the hot solution will be filled with a colloidal (greenish-yellow or reddish) precipitate as in Benedict's test. Because

[1] Benedict: *Jour. Biol. Chem.*, 5, 485, 1909: *Jour. Am. Med. Ass'n*, 57, 1193, 1911.

[2] Benedict's solution has the following composition:

Copper sulphate	17.3 grams.
Sodium citrate	173.0 grams.
Sodium carbonate (anhydrous)	100.0 grams.

Distilled water to make 1 liter.

With the aid of heat dissolve the sodium citrate and carbonate in about 800 c.c. of water. Pour (through a folded filter paper if necessary) into a glass graduate and make up to 850 c.c. Dissolve the copper sulphate in about 100 c.c. of water. Pour the carbonate-citrate solution into a large beaker or casserole and add the copper sulphate solution slowly, with constant stirring and make up to one liter. The mixed solution is ready for use and does not deteriorate upon long standing.

[3] Folin and McEllroy: *Jour. Biol. Chem.*, 33, 513, 1918.

[4] Folin-McEllroy Reagent.—It has been shown that alkaline phosphates may be used in place of tartrates or citrates to hold cupric hydroxide in solution. This reagent is based on that principle. Dissolve 100 g of sodium pyrophosphate, 30 g. of disodium phosphate and 50 g. of dry sodium carbonate in approximately 1 liter of water by the aid of a little heat. Dissolve separately 13 g. of copper sulphate in about 200 c.c. of water. Pour the copper sulphate solution into the phosphate-carbonate solution and shake.

of the sensitiveness of this test, when working with urine only a distinctly positive test obtained with the solution still *hot* is to be regarded as positive.

(e) Bismuth Reduction Test (Nylander).—To 5 c.c. of sugar solution in a test-tube add one-tenth its volume of Nylander's reagent[1] and heat for five minutes in a boiling water-bath.[2] The solution will darken if reducing sugar is present, and upon standing for a few moments a black color will appear.

This color is due to the precipitation of bismuth. If the test is made on a solution containing albumin this must be removed, by boiling and filtering, before applying the test, since with albumin a similar change of color is produced. Glucose when present to the extent of 0.08 per cent may be easily detected by this reaction (Rabe[3] claims that 0.01 per cent sugar may be so detected). Uric acid and creatinine which interfere with the Fehling's test do not interfere with the Nylander test. It is claimed by Bechold that the bismuth reduction tests give a negative reaction with solutions containing sugar when mercuric chloride or chloroform is present. Other observers[4] have failed to verify the inhibitory action of mercuric chloride and have shown that the inhibitory influence of chloroform may be overcome by raising the temperature of the urine to the boiling-point for a period of five minutes previous to making the test.

A positive bismuth reduction test is probably due to the following reactions:

$$(a)\quad Bi(OH)_2NO_3 + KOH \rightarrow Bi(OH)_3 + KNO_3.$$
$$(b)\quad 2Bi(OH)_3 - 3O \rightarrow Bi_2 + 3H_2O.$$

(f) Barfoed's Test.—Place about 5 c.c. of Barfoed's solution[5] in a test-tube and heat to boiling. Add glucose solution slowly, a few drops at a time, heating after each addition. Reduction is indicated by the formation of a red precipitate of cuprous oxide. If the precipitate does not form after boiling one-half minute[6] allow the tube to stand a few minutes and examine.

According to Welker[7] chlorides interfere pronouncedly with the reaction causing the formation of a green precipitate.

Barfoed's test is *not* a specific test for glucose as is frequently stated, but simply serves to detect *monosaccharides*. Disaccharides will also

[1] Nylander's reagent is prepared by digesting 2 grams of bismuth subnitrate and 4 grams of Rochelle salt in 100 c.c. of a 10 per cent potassium hydroxide solution. The reagent is then cooled and filtered.

[2] Hammarsten suggests that the mixture should be boiled 2–5 minutes (according to the sugar content) over a free flame and the tube then permitted to stand 5 minutes before drawing conclusions.

[3] Rabe: *Apoth. Ztg.*, 29, 554, 1914.

[4] Rehfuss and Hawk: *Journal of Biological Chemistry;* 7, 267, 1910; also Zeidlitz: *Upsala Lakäreforen Forh.*, N. F., 11, 1906.

[5] Barfoed's solution is prepared as follows: Dissolve 9 grams of neutral crystallized copper acetate in 100 c.c. of water and add 1.2 c.c. of 50 per cent acetic acid. This solution should be freshly made.

[6] Blake: *Jour. Am. Chem. Soc.*, 38, 1245, 1916.

[7] Welker: *Jour. Am. Chem. Soc.*, 37, 2227, 1915.

respond to the test, under proper conditions of acidity.[1] Also if the sugar solution is boiled sufficiently long, in contact with the reagent, to hydrolyze the disaccharide through the action of the acetic acid present in the Barfoed's solution a positive test results.[2] Barfoed's is a copper reduction test, but differs from Fehling's and other reduction tests in that the reduction is brought about in an acid solution. It is unsuited for the detection of sugar in urine.

(g) Picric Acid Test.—To 5 c.c. of the sugar solution add 2–3 c.c. of saturated picric acid solution and about 1 c.c. of 10 per cent Na_2CO_3. Warm. Note the development of a mahogany red color in the presence of glucose due to reduction of the picric acid with the formation of picramic acid:

$$\underset{\text{Picric Acid}}{C_6H_2 \cdot OH(NO_2)_3} \rightarrow \underset{\text{Picramic Acid}}{C_6H_2 \cdot OH \cdot NH_2(NO_2)_2}$$

This test has been made the basis of a method for the colorimetric determination of sugar in blood. See Chapter XVIII.

7. Alcoholic Fermentation.—Prepare 500 c.c. of a concentrated (10 per cent) solution of glucose, add a small amount of egg albumin or commercial peptone and introduce the mixture into a liter flask. Add yeast, and by means of a bent tube connect this flask with a second flask containing a solution of barium hydroxide protected from the air by a soda lime tube in the stopper (see Fig. 17). Place the flasks in a warm place and note the passage of gas bubbles into the barium hydroxide solution. As these gas bubbles (CO_2) enter, a white precipitate of barium carbonate will form. The glucose has been fermented according to the following equation:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$$

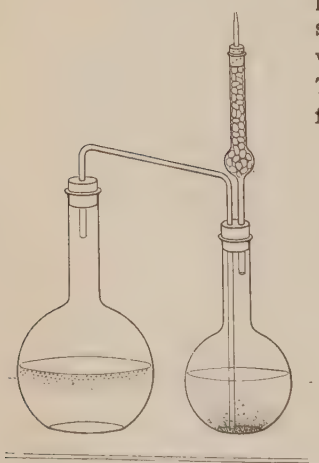


FIG. 17.—FERMENTATION APPARATUS.

FIG. 18.—IODOFORM. (*Autenrieth.*)

When the activity of the yeast has practically ceased decant the supernatant fluid, return it to the cleaned flask, connect with a condenser and distil. Catch

[1] Mathews and McGuigan: *Am. Jour. Physiol.*, 19, 175, 1907.
[2] Hinkle and Sherman: *Jour. Am. Chem. Soc.*, 29, 1744, 1907

the first portion of the distillate, which may be redistilled if its alcohol content is low, and test for alcohol by one of the following reactions:

(a) Iodoform Test.—Render 2–3 c.c. of the distillate alkaline with potassium hydroxide solution and add a few drops of iodine solution. Heat gently and note the formation of iodoform crystals. Examine these crystals under the microscope and compare them with those in Fig. 18.

(b) Aldehyde Test.—Place 5 c.c. of the distillate in a test-tube, add a few drops of potassium dichromate solution, $K_2Cr_2O_7$, and render it acid with dilute sulphuric acid. Boil the acid solution and note the odor of aldehyde changing to that of acetic acid.

8. Fermentation Test.—"Rub up" in a mortar about 20 c.c. of the sugar solution with a small piece of compressed yeast. Transfer the mixture to a saccharometer (shown in Fig. 19) and stand it aside in a warm place for about twelve hours. If the sugar is fermentable, alcoholic fermentation will occur and carbon dioxide will collect as a gas in the upper portion of the tube. On the completion of fermentation introduce a little potassium hydroxide solution into the graduated portion by means of a bent pipette, fill the bulb portion with water, place the thumb tightly over the opening in the apparatus and invert the saccharometer. Remembering that KOH has the power to absorb CO_2 how do you explain the result?[1] Filter some of the mixture. To 5 c.c. of the filtrate add several drops of a solution of iodine in potassium iodide (enough to give a yellow color to the whole mixture). Warm gently. Note iodoform odor and examine under microscope for crystals of iodoform (see Fig. 18). What does a positive test here indicate?

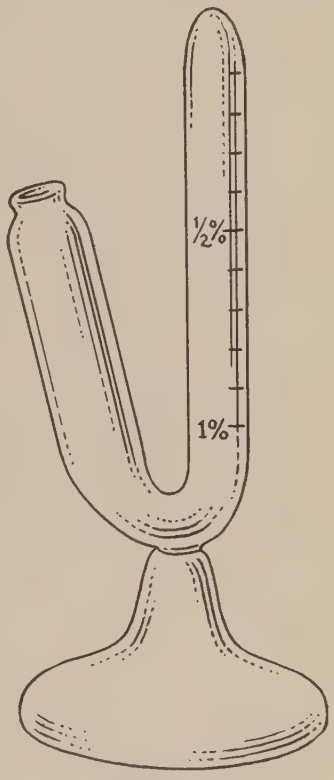


FIG. 19.—EINHORN SACCHAROMETER.

9. Formation of Caramel.—Gently heat a small amount of pulverized glucose in a test-tube. After the sugar has melted and turned brown, allow the tube to cool, add water and warm. The coloring matter produced is known as *caramel*.

10. Demonstration of Optical Activity.— A demonstration of the use of the polariscope, by the instructor, each student being required to take readings and compute the "specific rotation."

[1] The findings of Neuberg and associates[2] indicate that the liberation of carbon dioxide by yeast is not necessarily a criterion of the presence of sugar. The presence of an enzyme, called *carboxylase*, has been demonstrated in yeast which has the power of splitting off CO_2 from the carboxyl group of amino- and other aliphatic acids.

[2] Neuberg and associates: *Biochem. Zeitsch.*, 31, 170; 32, 323; 37, (60, 68,76), 1911.

Use of the Polariscope

For a detailed description of the different forms of polariscopes, the method of manipulation and the principles involved, the student is referred to any standard text-book of physics. A brief description follows. A ray of ordinary light vibrates in every direction. If such a ray is caused to pass through a "polarizing" Nicol prism it is resolved into *two rays* vibrating in single planes perpendicular to each other. Such light is said to be *polarized*. By reflecting one of the rays out of the field of vision light is obtained vibrating in *one plane only*. Many organic substances (sugar, proteins, etc.) have the power of twisting or rotating this plane of polarized light, the extent to which

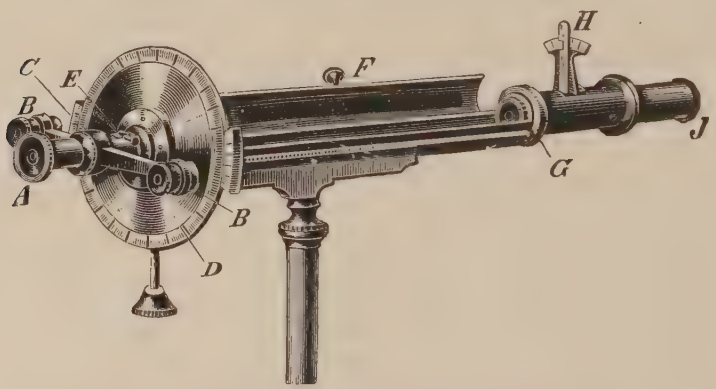


Fig. 20.—One Form of Laurent Polariscope.

B Microscope for reading the scale; *C*, a vernier; *E*, position of the analyzing Nicol prism; *H*, polarizing Nicol prism in the tube below this point.

the plane is rotated depending upon the number of molecules which the polarized light passes. Substances which possess this power are said to be "optically active." The *specific rotation* of a substance is the rotation expressed in degrees which is afforded by 1 gram of substance dissolved in 1 c.c. of water in a tube one decimeter in length. The specific rotation, $(\alpha)_D$, may be calculated by means of the following formula:

$$(\alpha)_D = \frac{\alpha}{p.l},$$

in which

D = sodium light.
α = observed rotation in degrees.
p = grams of substance dissolved in 1 c.c. of liquid.
l = length of the tube in decimeters.

If the specific rotation has been determined and it is desired to ascertain the per cent of the substance in solution, this may be obtained by the use of the following formula,

$$p = \frac{\alpha}{(\alpha)_D l}$$

The value of p multiplied by 100 will be the percentage of the substance in solution.

SPECIFIC ROTATIONS OF MORE COMMON CARBOHYDRATES[1]

d-Glucose	+ 52.5°	Sucrose	+ 66.5°
d-Fructose[2]	− 92.3°	Lactose	+ 52.5°
d-Galactose	+ 81.5°	Maltose	+ 137.0°
d-Mannose	+ 14.2°	Raffinose	+ 104.0°
l-Arabinose	+ 104.5°	Dextrin	+ 195.0°
l-Xylose	+ 19.0°	Starch (soluble)	+ 196.0°
Rhamnose	+ 9.0°	Glycogen	+ 197.0°

An instrument by means of which the extent of the rotation may be determined is called a *polariscope* or *polarimeter*. Such an instrument

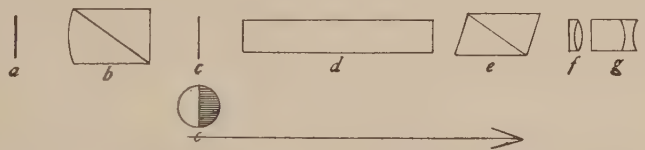


FIG. 21.—DIAGRAMMATIC REPRESENTATION OF THE COURSE OF THE LIGHT THROUGH THE LAURENT POLARISCOPE. (The direction is reversed from that of Fig. 20, p. 80.)

a, Bichromate plate to purify the light; *b*, the polarizing Nicol prism; *c*, a thin quartz plate covering one-half the field and essential in producing a second polarized plane; *d*, tube to contain the liquid under examination; *e*, the analyzing Nicol prism; *f* and *g*, ocular lenses.

designed especially for the examination of sugar solutions is termed a *saccharimeter* or *polarizing saccharimeter*. The form of polariscope in Fig. 20, page 80, consists essentially of a long barrel provided with a Nicol prism at either end (Fig. 21). The solution under examination is contained in a tube which is placed between these two prisms. At the front end of the instrument is an adjusting eyepiece for focusing and a large recording disc which registers in degrees and fractions of a degree. The light is admitted into the far end of the instrument and is polarized by passing through a Nicol prism. This polarized ray then

[1] The specific rotation varies with the temperature and concentration of the solution. The figures here given are for concentrations of about 10 per cent and temperatures of about 20°C. Fresh solutions may give markedly different values due to mutarotation, the figures here given representing the constant values obtained on standing.

[2] It should be noted that *d*- and *l*- do not represent the *direction* of optical rotation, but indicate a chemical relationship to other dextrorotatory or levo-rotatory sugars.

traverses the column of liquid within the tube mentioned above and if the substance is optically active the plane of the polarized ray is rotated to the right or left. Bodies rotating the ray to the right are called *dexro-rotatory* and those rotating it to the left *levo-rotatory*.

Within the apparatus is a disc which is so arranged as to be without lines and uniformly light at zero. Upon placing the optically active substance in position, however, the plane of polarized light is rotated or turned and it is necessary to rotate the disc through a certain number of degrees in order to secure the normal conditions, *i.e.*, "without lines

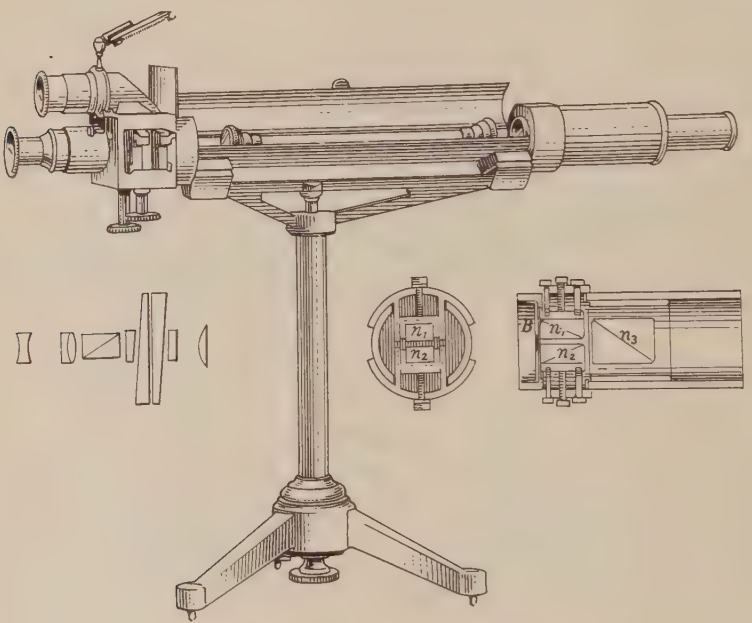


FIG. 22.—POLARISCOPE (SCHMIDT AND HÄNSCH MODEL).

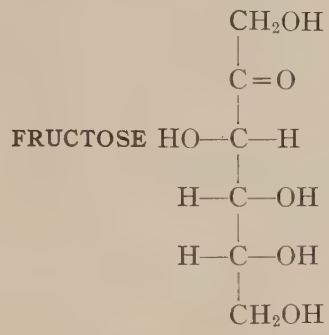
and uniformly light." The difference between this reading and the *zero* is α or the observed rotation in degrees.

Sugar solutions (glucose, levulose, lactose, maltose, but not sucrose) when freshly prepared possess a changing rotation, so called *mutarotation*. For this reason such solutions before polariscopic examination should be allowed to stand over night, heated to 100°C. and then cooled, or treated with a drop of ammonia followed by a drop of acid. This brings about an equilibrium between the α and β forms possessing different rotations and of which ordinary glucose is a mixture.

Polarizing saccharimeters are also constructed by which the percentage of sugar in solution is determined by making an observation and multiplying the value of each division on a horizontal sliding scale

by the value of the division expressed in terms of dextrose. This factor may vary according to the instrument.

"Optical" methods embracing the determination of the optical activity are being utilized in recent years in many "quantitative" connections.[1]



As already stated, fructose, sometimes called levulose or fruit sugar, occurs widely disseminated throughout the plant kingdom in company with glucose. Its preparation from the inulin of the Jerusalem artichoke promises to become of commercial importance. Although it is a ketose it nevertheless reduces metallic oxides in alkaline solution due to the presence of the terminal group $CO \cdot CH_2OH$. For the same reason monohydroxyacetone ($CH_3 \cdot CO \cdot CH_2OH$) also reduces such solutions although acetone ($CH_3 \cdot CO \cdot CH_3$) does not. The reducing power of fructose is somewhat weaker than that of dextrose. Fructose does not ordinarily occur in the urine in diabetes mellitus, but has been found in exceptional cases. With phenylhydrazine it forms the same osazone as glucose. With methylphenylhydrazine, fructose forms a characteristic methylphenyl fructosazone. Fructose, like glucose, exhibits the power of mutarotation, due to the fact that in solution it exists in two forms as α and β lactones, the latter being the more reactive. The specific rotation of the fresh solution, -133.5°, drops to -92.3° on standing, or in the presence of a trace of alkali.

(For a further discussion of fructose see the section on Hexoses, page 69.)

Experiments on Fructose

1–6. Repeat Solubility, Benedict's, Phenylhydrazine, Barfoed's, Nylander's, and Fermentation tests as given under Glucose, pages 71–79.

[1] Abderhalden and Schmidt: "Determination of blood content by means of the optical method," *Zeit. physiol. Chem.*, 66, 120, 1910; also C. Neuberg: "Determination of nucleic acid cleavage by polarization," *Biochemische Zeitschrift*, 30, 505, 1911.

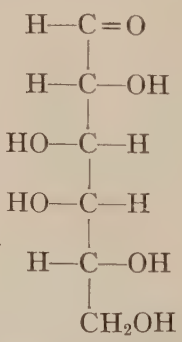
7. Resorcinol-Hydrochloric Acid Reaction (Seliwanoff).—To 5 c.c. of Seliwanoff's reagent[1] in a test-tube add a few drops of a fructose solution and heat the mixture to boiling. A positive reaction is indicated by the production of a red color and the separation of a brown-red precipitate. The latter may be dissolved in alcohol to which it will impart a striking red color.

If the boiling be prolonged a similar reaction may be obtained with solutions of glucose or maltose. This has been explained[2] in the case of glucose as due to the transformation of the glucose into fructose by the catalytic action of the hydrochloric acid. The precautions necessary for a positive test for levulose are as follows: The concentration of the hydrochloric acid must not be more than 12 per cent. The reaction (red color) and the precipitate must be observed after not more than 20–30 seconds boiling. Glucose must not be present in amounts exceeding 2 per cent. The precipitate must be soluble in alcohol with a bright red color.

8. Borchardt's Reaction.—To about 5 c.c. of a solution of fructose in a test-tube add an equal volume of 25 per cent hydrochloric acid and a few crystals of resorcinol. Heat to boiling and after the production of a red color, cool the tube under running water and transfer to an evaporating dish or beaker. Make the mixture slightly alkaline with *solid* potassium hydroxide, return it to a test-tube, add 2–3 c.c. of acetic ether and shake the tube vigorously. In the presence of levulose, the acetic ether is colored yellow. (For further discussion of the test see Chapter XXVIII.)

9. Formation of Methylphenylfructosazone.—To a solution of 1.8 grams of levulose in 10 c.c. of water add 4 grams[3] of methylphenylhydrazine and enough alcohol to clarify the solution. Introduce 4 c.c. of 50 per cent acetic acid and heat the mixture for 5–10 minutes on a boiling water-bath.[4] On standing 15 minutes at room temperature, crystallization begins and is complete in two hours. By scratching the sides of the flask or by inoculation, the solution quickly congeals to form a thick paste of reddish-yellow silky needles. These are the crystals of *methylphenylfructosazone*. They may be recrystallized from hot 95 per cent alcohol and melt at 153°C.

GALACTOSE



[1] Seliwanoff's reagent may be prepared by dissolving 0.05 gram of resorcinol in 100 c.c. of dilute (1 : 2) hydrochloric acid.

[2] Koenigsfeld: *Bioch. Zeit.*, 38, 311, 1912.

[3] 3.66 grams if absolutely pure.

[4] Longer heating is to be avoided.

Galactose occurs with glucose as one of the products of the hydrolysis of lactose.[1] It is dextro-rotatory, forms an osazone with phenylhydrazine and ferments slowly with yeast. Upon oxidation with nitric acid galactose yields mucic acid, thus differentiating this monosaccharide from glucose and fructose. Lactose also yields mucic acid under these conditions. The mucic acid test may be used in urine examination to differentiate lactose and galactose from other reducing sugars. Galactose exhibits the property of mutarotation in solution.

Experiments on Galactose

1. Phloroglucinol-Hydrochloric Acid Reaction (Tollens).—To equal volumes of galactose solution and hydrochloric acid (sp. gr. 1.09) add a little phloroglucinol, and heat the mixture on a boiling water-bath. Galactose, pentose and glycuronic acid will be indicated by the appearance of a red color. Galactose may be differentiated from the two latter substances in that its solutions exhibit no absorption bands upon spectroscopical examination.

2. Mucic Acid Test.—Treat 100 c.c. of the solution containing galactose with 20 c.c. of concentrated nitric acid (sp. gr. 1.4) and evaporate the mixture in a broad, shallow glass vessel on a boiling water-bath until the volume of the mixture has been reduced to about 20 c.c. At this point the fluid should be clear, and a fine white precipitate of mucic acid should form.

If the percentage of galactose present is low it may be necessary to cool the solution and permit it to stand for some time before the precipitate will form. It is impossible to differentiate between galactose and lactose by this test, but the reaction serves to differentiate these two sugars from all other reducing sugars. Differentiate lactose from galactose by means of Barfoed's test (page 77).

3. Phenylhydrazine Reaction.—Make the test according to directions given under Glucose, 3, page 72.

PENTOSES, $C_5H_{10}O_5$

In plants, and more particularly in certain gums, very complex carbohydrates, called pentosans (see page 99), occur. These pentosans through hydrolysis by acids may be transformed into pentoses. Pentoses do not ordinarily occur in the animal organism, but have been found in the urine of morphine addicts and others, their occurrence sometimes being a persistent condition without known cause. They may be obtained from the hydrolysis of nucleoproteins being present in the nucleic acid complex of the molecule. Pentoses are non-fermentable, have strong reducing power and form osazones with phenylhydrazine. Pentoses are an important constituent of the dietary of herbivorous animals. Glycogen is said to be formed after the ingestion

[1] For preparation of pure galactose from lactose see Clark: *Jour. Biol. Chem.*, 47, 1, 1921.

of these sugars containing five oxygen atoms. This, however, has not been conclusively proven. On distillation with strong hydrochloric acid pentoses and pentosans yield furfurol, which can be detected by its characteristic red reaction with aniline-acetate paper.

ARABINOSE, $CH_2OH(CHOH)_3CHO$

Arabinose is one of the most important of the pentoses. The *l*-arabinose may be obtained from gum arabic, plum or cherry gum by boiling for 10 minutes with concentrated hydrochloric acid. This pentose is dextro-rotatory, forms an osazone and has reducing power, but does not ferment. The *i*-arabinose has been isolated from the urine and yields an osazone which melts at 166°–168°C.

Experiments on Arabinose

1. Orcinol-Hydrochloric Acid Reaction (Bial).[1]—To 5 c.c. of Bial's reagent[2] in a test-tube add 2–3 c.c. of the arabinose solution and heat the mixture gently until the first bubbles rise to the surface. Immediately or upon cooling the solution becomes green and a flocculent precipitate of the same color may form. (For further discussion see Chapter XXVIII.) The test may also be performed by adding the pentose to the hot reagent.

Menthol, kreosotal, etc. do not interfere with this test. Sachs[3] has offered suggestions as to modification of the test in order to avoid confusion with glycuronic acid.

2. Phloroglucinol-Hydrochloric Acid Reaction (Tollens).—To equal volumes of arabinose solution and hydrochloric acid (sp. gr. 1.09) add a little phloroglucinol and heat the mixture on a boiling water-bath. Galactose, pentose or glycuronic acid will be indicated by the appearance of a red color. To differentiate between these bodies make a spectroscopic examination and look for the absorption band between *D* and *E* given by pentoses and glycuronic acid. Differentiate between the two latter bodies by the melting-points of their osazones.

Compare the reaction with that obtained with galactose (page 85).

3. Phenylhydrazine Reaction.—Make this test on the arabinose solution according to directions given under Glucose, 3, page 72.

XYLOSE, $CH_2OH(CHOH)_3CHO$

[1] Bial: *Deut. med. Woch.*, 28, 252, 1902, and *Berl. klin. Woch.*, No. 18, 1903.
[2] Orcinol.....1.5 gram.
HCl (conc.).....500 grams.
Ferric chloride (10 per cent) 20–30 drops.
[3] Sachs: *Bioch. Zeit.*, 1, 383, 1906, and 2, 245, 1906.

Xylose, or wood sugar, is obtained by boiling wood gums with dilute acids as explained under Arabinose, (see above). It is dextro-rotatory, forms an osazone and has reducing power, but does not ferment.

Experiments on Xylose

1–3. Same as for arabinose (see page 86).

RHAMNOSE, $C_6H_{12}O_5$

Rhamnose or methyl-pentose is an example of a true carbohydrate which does not have the H and O atoms present in the proportion to form water. It has been found that rhamnose when ingested by rabbits or hens has a positive influence upon the formation of glycogen in those organisms.

DISACCHARIDES, $C_{12}H_{22}O_{11}$

The disaccharides as a class may be divided into two rather distinct groups. The first group would include those disaccharides which are found in nature as such, *e.g.*, *sucrose* and *lactose*, and the second group would include those disaccharides formed in the hydrolysis of more complex carbohydrates, *e.g.*, *maltose* and *iso-maltose*.

The disaccharides have the general formula $C_{12}H_{22}O_{11}$, to which, in the process of hydrolysis, a molecule of water is added causing the single disaccharide molecule to split into two monosaccharide (hexose) molecules. The products of the hydrolysis of the more common disaccharides are as follows:

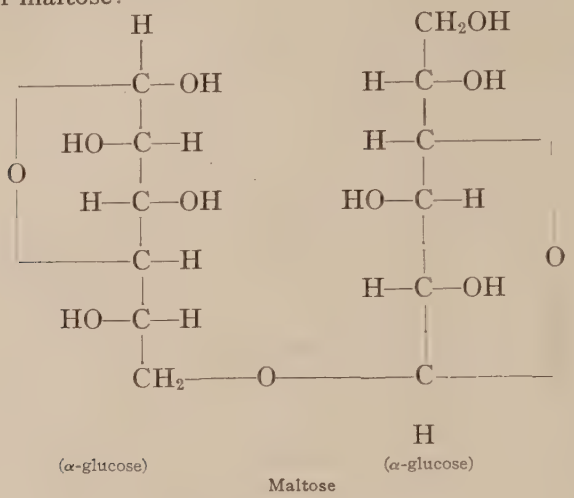
Maltose = glucose + glucose.
Lactose = glucose + galactose.
Sucrose = glucose + fructose.

All of the more common disaccharides *except sucrose* have the power of reducing certain metallic oxides in alkaline solution, notably those of copper and bismuth. This reducing power is due to the presence of the *potential aldehyde group* (—CHO) in the sugar molecule.

MALTOSE, $C_{12}H_{22}O_{11}$

Maltose or malt sugar is formed in the hydrolysis of starch through the action of an enzyme, *vegetable amylase* (*diastase*), contained in sprouting barley or malt. Certain enzymes in the saliva and in the pancreatic juice may also cause a similar hydrolysis. Maltose is also an intermediate product of the action of dilute mineral acids upon starch. It is strongly dextro-rotatory, reduces metallic oxides in alkaline solution and is fermentable by yeast after being inverted (see Polysaccharides, page 91) by the enzyme *maltase* of the yeast. In common with the other disaccharides, maltose may be hydrolyzed with the formation of two molecules of monosaccharide. In this instance the products are two

molecules of glucose. With phenylhydrazine maltose forms an osazone, *maltosazone*. The following formula represents the probable structure of maltose:



Maltose

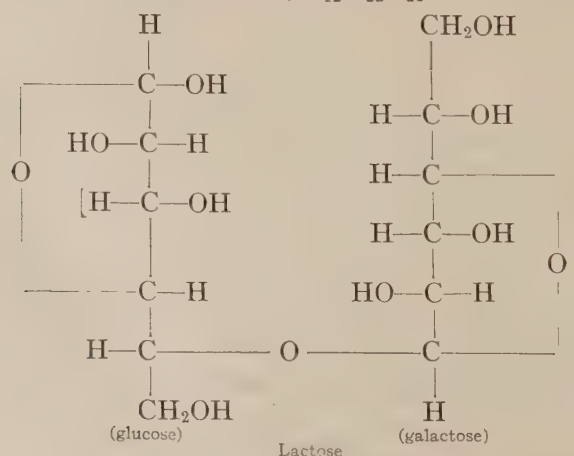
Experiments on Maltose

1–6. Repeat Solubility, Benedict's, Nylander's, Phenylhydrazine, Barfoed's and Fermentation tests as given under Glucose, pages 71–79.

Iso-maltose, $C_{12}H_{22}O_{11}$

Iso-maltose, an isomeric form of maltose, is formed along with maltose, by the action of diastase upon starch paste, and also by the action of hydrochloric acid upon glucose. It also occurs with maltose as one of the products of salivary digestion. It is dextro-rotatory and with phenylhydrazine gives an osazone which is characteristic. Iso-maltose is very soluble and reduces the oxides of bismuth and copper in alkaline solution. Pure iso-maltose is probably only slightly fermentable.

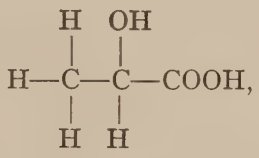
Lactose, $C_{12}H_{22}O_{11}$



Lactose

Lactose or milk sugar occurs ordinarily only in milk, but has often been found in the urine of women during pregnancy and lactation. It may also occur in the urine of normal persons after the ingestion of unusually large amounts of lactose in the food. It has a strong reducing power, is dextro-rotatory and forms an osazone with phenylhydrazine. Upon hydrolysis lactose yields one molecule of glucose and one molecule of galactose.

In the souring of milk the *Lactobacillus acidophilus* or *Streptococcus lacticus* and certain other micro-organisms bring about lactic acid fermentation by transforming the lactose of the milk into lactic acid,



and alcohol. This same reaction may occur in the alimentary canal as the result of the action of B. *acidophilus* and certain other organisms. In the preparation of kephyr and koumyss the lactose of the milk undergoes alcoholic fermentation, through the action of ferments other than yeast, and at the same time lactic acid is produced. Lactose and galactose yield *mucic acid* on oxidation with nitric acid. This fact is made use of in urine analysis to facilitate the differentiation of these sugars from other reducing sugars. Mucic acid is $COOH(HCOH)_4COOH$.

Lactose is *not* fermentable by ordinary baker's yeast (Chap. XXVIII). Mathews[1] has suggested an easy way to differentiate and determine lactose in the presence of glucose based on reduction before and after fermentation with yeast.

Experiments on Lactose

1–6. Repeat Solubility, Benedict's, Phenylhydrazine, Barfoed's, Nylander's and Fermentation tests as given under Glucose, pages 71–79.

7. Mucic Acid Test.—Treat 100 c.c. of the solution containing lactose with 20 c.c. of concentrated nitric acid (sp. gr. 1.4) and evaporate the mixture in a broad, shallow glass vessel on a boiling water-bath, until the volume of the mixture has been reduced to about 20 c.c. At this point the fluid should be clear, and a fine white precipitate of mucic acid should form.

If the percentage of lactose present is low it may be necessary to cool the solution and permit it to stand for some time before the precipitate will appear. It is impossible to differentiate between lactose and galactose by this test, but the reaction serves to differentiate these two sugars from all other reducing sugars.

[1] Mathews: *Jour. Am. Med. Ass'n.*, 75, 1568, 1920.

Differentiate lactose from galactose by means of Barfoed's test, page 77.

SUCROSE, $C_{12}H_{22}O_{11}$

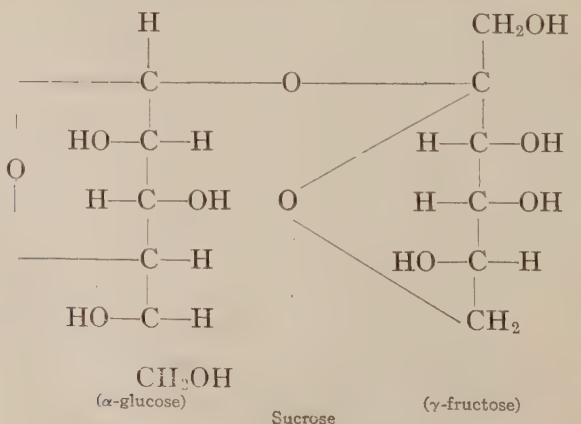
Sucrose, also called saccharose or cane sugar, is one of the most important of the sugars and occurs very extensively distributed in plants, particularly in the sugar cane, sugar beet, sugar millet and in certain palms and maples.

Sucrose is dextro-rotatory and upon hydrolysis, as before mentioned, the molecule of sucrose takes on a molecule of water and breaks down into two molecules of monosaccharide. The monosaccharides formed in this instance are glucose and fructose. This is the reaction:

$$\underset{\text{Sucrose}}{C_{12}H_{22}O_{11}} + H_2O \rightarrow \underset{\text{Glucose}}{C_6H_{12}O_6} + \underset{\text{Fructose}}{C_6H_{12}O_6}.$$

This process is called *inversion* and may be produced by bacteria, enzymes, and certain weak acids. After this inversion the previously strongly dextro-rotatory solution becomes levo-rotatory. This is due to the fact that the fructose molecule is more strongly levo-rotatory than the glucose molecule is dextro-rotatory. The product of this inversion is called *invert sugar*.

Sucrose does *not* reduce metallic oxides in alkaline solution and forms *no* osazone with phenylhydrazine. Prolonged boiling in the presence of an *acid* phenylhydrazine solution will, however, hydrolyze the sucrose and cause the formation of glucosozone and fructosozone. It is not fermentable directly by yeast, but must first be *inverted* by the enzyme *sucrase* (*invertase* or *invertin*) contained in the yeast. The probable structure of sucrose may be represented by the following formula. Note the absence of any potential ketone or aldehyde group. Upon inversion, the active forms of glucose and fructose indicated in the formula are rapidly transformed by mutarotation to the more stable modifications.



Sucrose

Experiments on Sucrose

1–6. Repeat Solubility, Benedict's, Nylander's, Barfoed's, Phenylhydrazine and Fermentation tests according to the directions given under Glucose, pages 71–79.

7. Inversion of Sucrose.—To 25 c.c. of sucrose solution in a beaker add 5 drops of concentrated H_2SO_4 and boil one minute. Cool the solution and render neutral with saturated barium hydroxide. Filter off the precipitate of barium sulphate and upon the resulting fluid repeat the phenylhydrazine, Benedict, Nylander's and Barfoed's reactions as given under Glucose, pages 72, 76, and 77; and the Resorcinol-Hydrochloric Acid Reaction (Seliwanoff), as given under Fructose, page 84. Explain the results.

TRISACCHARIDES, $C_{18}H_{32}O_{16}$

RAFFINOSE

This trisaccharide, also called melitose, or melitriose occurs in cotton seed, Australian manna, and in the molasses from the preparation of beet sugar. It is dextro-rotatory, does not reduce Benedict's solution, and is only partly fermentable by yeast.

Raffinose may be hydrolyzed by weak acids the same as the polysaccharides are hydrolyzed, the products being fructose and melibiose. Further hydrolysis of the melibiose yields glucose and galactose. Raffinose may also be hydrolyzed by the enzyme raffinase, occurring in certain bacteria and yeasts.[1]

POLYSACCHARIDES, $(C_6H_{10}O_5)_x$

In general the polysaccharides are amorphous bodies, a few, however, are crystallizable. Through the action of certain enzymes or weak acids the polysaccharides may be hydrolyzed with the formation of monosaccharides. As a class the polysaccharides are quite insoluble and are non-fermentable until inverted. By inversion is meant the hydrolysis of disaccharide or polysaccharide sugars to form monosaccharides, as indicated in the following equations:

$$(a) \qquad C_{12}H_{22}O_{11}+H_2O \rightarrow 2C_6H_{12}O_6.$$

$$(b) \qquad C_6H_{10}O_5+H_2O \rightarrow C_6H_{12}O_6.$$

STARCH, $(C_6H_{10}O_5)_x$

Starch is widely distributed throughout the vegetable kingdom, occurring in grains, fruits, and tubers. It occurs in granular form, the microscopical appearance being typical for each individual starch. The granules, which differ in size according to the source, contain,

[1] Kuriyama and Mendel: *Jour. Biol. Chem.*, 31, 125, 1917.

according to recent work,[1] at least three principal ingredients, *amylocellulose* forming the cell walls, making up about 10 per cent of the starch granules and not reacting with iodine; *amylose*, comprising about 70 per cent of the granules and giving a blue color with iodine; and *amylopectin*, the substance giving the high viscosity to starch pastes, making up about 20 per cent of the granules, and giving no color with iodine. Ordinary starch is insoluble in cold water, but if boiled with water the cell walls are ruptured and *starch paste* results. If starch is ground in a pebble mill it will no longer form a paste and 60 per cent of it dissolves in cold water.[2] In general starch gives a blue color with iodine.

Starch is acted upon by amylases, *e.g.*, salivary amylase (*ptyalin*) and pancreatic amylase (*amylopsin*), with the formation of *soluble starch, erythro-dextrin, achroo-dextrins, and maltose* (see Salivary Digestion, page 106). Maltose is the principal end-product of this enzyme action. Upon boiling a starch solution with a dilute mineral acid a series of similar bodies is formed, but under these conditions *glucose* is the principal end-product. The catalytic agent here is the hydrogen ion.

Soluble starch may be prepared by the action of dilute hydrochloric acid upon starch.[3]

Experiments on Starch

1. Preparation of Potato Starch.—Pare a raw potato, comminute it upon a fine grater, mix with water, and "whip up" the pulped material vigorously before straining it through cheese cloth or gauze to remove the coarse particles. The starch rapidly settles to the bottom and can be washed by repeated decantation. Allow the compact mass of starch to drain thoroughly and spread it out on a watch glass to dry in the air. If so desired this preparation may be used in the experiments which follow.

2. Microscopical Examination.—Examine microscopically the granules of the various starches submitted and compare them with those shown in Figs. 23–33 page 94. The suspension of the granules in a drop of water will facilitate the microscopical examination.

3. Solubility.—Try the solubility of one form of starch in each of the ordinary solvents (see page 71). If uncertain regarding the solubility in any reagent, filter and test the filtrate with iodine solution as given under 5 below. The pro-

[1] Blake: *Jour. Am. Chem. Soc.*, 38, 1245, 1916; 39, 315, 1917. Maquenne and Roux: *Ann. Chim. Phys.*, 9, 179, 1906.

[2] Alsberg and Perry: *Proc. Soc. Exp. Biol. Med.*, 22, 60, 1924.

[3] Small: *J. Am. Chem. Soc.*, 41, 113, 1919. Suspend 3 gm. raw potato starch in 100 c.c. of redistilled 95 per cent alcohol, add 0.75 c.c. of concentrated HCl and heat on a boiling water bath with a reflux condenser for exactly ten minutes. Add at once an amount of normal sodium bicarbonate solution just sufficient to neutralize 0.75 c.c. of the acid using methyl orange as indicator. Decant through a filter and wash with several portions of alcohol. Dry at room temperature, sieve and preserve.

duction of a blue color would indicate that the starch had been dissolved by the solvent.

4. Iodine Test.—Place a few granules of starch in one of the depressions of a porcelain test-tablet and treat with a drop of a dilute solution of iodine in potassium iodide. The granules are colored blue due to the formation of so-called iodide of starch. The amylo-cellulose of the granule is not stained as may be seen by examining microscopically.

5. Iodine Test on Starch Paste.[1]—Repeat the iodine test using the starch paste. Place 2–3 c.c. of starch paste[2] in a test-tube, add a drop of the dilute iodine solution and observe the production of a blue color. Heat the tube and note the disappearance of the color. It reappears on cooling.

In similar tests note the influence of alcohol and of alkali upon the so-called iodide of starch.

The composition of the iodide of starch is not definitely known. In performing this test the solution must always be neutral or acid in reaction.

6. Benedict's Test.—On starch paste (see page 76).

7. Does Iodine React with "Dry" Starch?[3]—Place a sample of ground starch[4] on a glass slide and spread it out in a thin layer with a spatula. Put the slide in a small, empty desiccator. On another slide place enough iodine crystals to saturate the desiccator with iodine vapor. Close the desiccator and allow it to stand 24 hours at room temperature. At the end of this period the starch should be brown due to condensed or adsorbed iodine, but no blue color should be evident.

Now introduce into the desiccator sufficient water to saturate the system, using care that the fluid does not come into contact with the starch or iodine. Close the desiccator and examine at half hour intervals for two hours and again at the end of 24 hours. A trace of blue should appear on the starch in less than a half hour and in 24 hours the starch should be deep blue in color.

8. Hydrolysis of Starch.—Place about 25 c.c. of starch paste in a small beaker, add 10 drops of concentrated HCl, and boil. By means of a small pipette, at the end of each minute, remove a drop of the solution to the test-tablet and make the regular iodine test. As the testing proceeds the blue color should gradually fade and finally disappear. At this point, after cooling and neutralizing with solid KOH, Benedict's test (see page 76) should give a positive result due to the formation of a reducing sugar from the starch. Make the phenylhydrazine test upon some of the hydrolyzed starch. What sugar has been formed?

9. **Influence of Tannic Acid.**—Add an excess of tannic acid solution to a small amount of starch paste in a test-tube. The liquid will become strongly opaque and ordinarily a yellowish-white precipitate is produced. Compare this result with the result of the similar experiment on dextrin (page 97).

[1] *Preparation of Starch Paste.*—Grind 2 grams of starch powder in a mortar with a small amount of cold water. Bring 200 c.c. of water to the boiling-point and add the starch mixture from the mortar with continuous stirring. Bring again to the boiling-point and allow it to cool. This makes an approximate 1 per cent starch paste which is a very satisfactory strength for general use.

[2] For this particular test a starch paste of very satisfactory strength may be made by mixing 1 c.c. of a 1 per cent starch paste with 100 c.c. of water.

[3] Field: *Proc. Soc. Exp. Biol. Med.*, 23, 310, 1926.

[4] Alsberg and Rask: *Cereal Chem.*, 1, 7, 1924.
Alsberg and Perry: *Proc. Soc. Exp. Biol. Med.*, 22, 60, 1924.

FIG. 23.—POTATO.

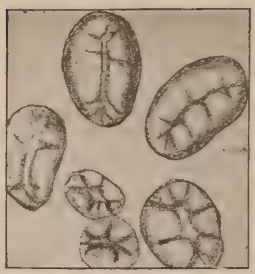


FIG. 24.—BEAN.

FIG. 25.—ARROWROOT.

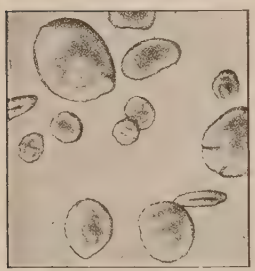


FIG. 26.—RYE.

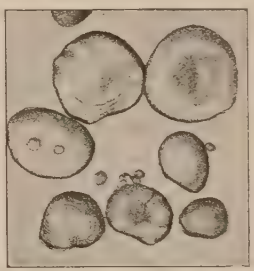


FIG. 27.—BARLEY.

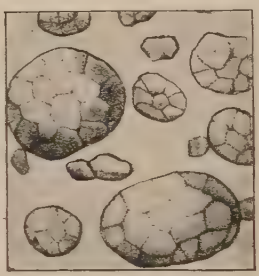


FIG. 28.—OAT.

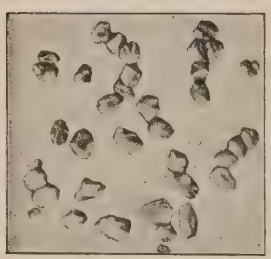


FIG. 29.—BUCKWHEAT.

FIG. 30.—MAIZE.

FIG. 31.—RICE.

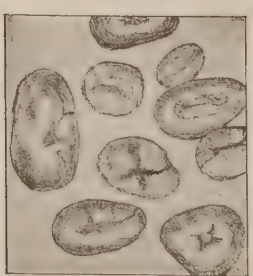


FIG. 32.—PEA.

FIG. 33.—WHEAT.

STARCH GRANULES FROM VARIOUS SOURCES. (*Leffmann and Beam*)

10. Diffusibility of Starch Paste.—Test the diffusibility of starch paste through animal membrane, parchment paper or collodion, making a dialyzer like one of the models shown in Fig. 16, page 74.

INULIN, $(C_6H_{10}O_5)_x$

Inulin is a polysaccharide which may be obtained as a white, odorless, tasteless powder from the tubers of the artichoke, elecampane, or dahlia. It has also been prepared from the roots of chicory, dandelion, and burdock. It is very slightly soluble in cold water and quite easily soluble in hot water. In cold alcohol of 60 per cent or over it is practically insoluble. Inulin gives a negative reaction with iodine solution. The "yellow" color reaction with iodine mentioned in many books is doubtless merely the normal color of the iodine solution. It is very difficult to prepare inulin which does not reduce Benedict's solution slightly. This reducing power may be due to an impurity. Practically all commercial preparations of inulin possess considerable reducing power.

Inulin is levo-rotatory and upon hydrolysis by acids or by the enzyme *inulase* it yields the monosaccharide fructose which readily reduces Benedict's solution. The preparation of fructose by hydrolysis of the inulin of the Jerusalem artichoke promises to become of commercial importance. The ordinary amylolytic enzymes occurring in the animal body do not digest inulin. A small part of the ingested inulin may be hydrolyzed by the acid gastric juice, but Lewis[1] has shown that "the value of inulin as a significant source of energy in human dietaries must be questioned."

Experiments on Inulin

1. Solubility.—Try the solubility of inulin powder in hot and cold water and alcohol. If uncertain regarding the solubility in any reagent, filter and neutralize the filtrate if it is alkaline in reaction. Add a drop of concentrated hydrochloric acid to the filtrate and boil it for one minute. Render the solution neutral or slightly alkaline with solid potassium hydroxide and try Benedict's test. What is the significance of a positive Benedict's test in this connection?

2. Iodine Test.—(a) Place 2–3 c.c. of the inulin solution in a test-tube and add a drop of dilute iodine solution. What do you observe?

(b) Place a small amount of inulin powder in one of the depressions of a test-tablet and add a drop of dilute iodine solution. Is the effect any different from that observed above?

3. Molisch's Reaction.—Repeat this test according to directions given under Glucose, 2, page 71.

4. Benedict's Test.—Make this test on the inulin solution according to the instructions given under Glucose, page 76. Is there any reduction?[2]

5. Hydrolysis of Inulin.—Place 5 c.c. of inulin solution in a test-tube, add a drop of concentrated hydrochloric acid and boil it for one minute. Now cool the solution, neutralize it with concentrated KOH and test the reducing action

[1] Lewis: *Journal American Medical Ass'n*, 58, 1176, 1912.
[2] See the discussion of the properties of inulin, above.

of 1 c.c. of the solution upon 1 c.c. of Benedict's solution. Also try the Resorcinol-Hydrochloric Acid reaction as given on p. 84. Explain the result.[1]

GLYCOGEN, $(C_6H_{10}O_5)_x$

(For discussion and experiments see Muscular Tissue, Chapter XXIV.)

LICHENIN, $(C_6H_{10}O_5)_x$

Lichenin may be obtained from *Cetraria islandica* (Iceland moss). It forms a difficultly soluble jelly in cold water and an opalescent solution in hot water. It is optically inactive and gives no color with iodine. Upon hydrolysis with dilute mineral acids lichenin yields dextrins and glucose. It is said to be most nearly related chemically to starch. Saliva, pancreatic juice, malt diastase, and gastric juice have no noticeable action on lichenin.

DEXTRIN, $(C_6H_{10}O_5)_x$

The dextrins are the bodies formed midway in the stages of the hydrolysis of starch by weak acids or an enzyme. They are amorphous bodies which are easily soluble in water, acids, and alkalis, but are insoluble in alcohol or ether. Dextrins are dextro-rotatory and are not fermentable by yeast.

The dextrins may be hydrolyzed by dilute acids to form *glucose* and by amylases to form *maltose*. With iodine one form of dextrin (erythro-dextrin) gives a red color. Their power to reduce Benedict's solution is questioned. The lower members of the dextrin series probably reduce.

Experiments on Dextrin

1. Solubility.—Test the solubility of pulverized dextrin in hot and cold water. Dextrin forms a clear solution in hot water, distinguishing it from glycogen which gives an opalescent solution.

2. Iodine Test.—Place a drop of dextrin solution in one of the depressions of the test-tablet and add a dilute solution of iodine in potassium iodide. A red color results due to the formation of the red iodide of dextrin. Ordinary dextrin preparations contain some starch and in the presence of starch it is necessary to have an excess of iodine present. If the reaction is not sufficiently pronounced make a stronger solution from pulverized dextrin and repeat the test. The solution should be slightly acid to secure the best results.

Make proper tests to show that the red iodide of dextrin is influenced by heat, alkali, and alcohol in a similar manner to the blue iodide of starch (see page 93).

[1] If the inulin solution gave a positive Benedict test in the last experiment it will be necessary to check the hydrolysis experiment as follows: To 5 c.c. of the inulin solution in a test-tube add one drop of concentrated hydrochloric acid, neutralize with concentrated KOH solution and test the reducing action of 1 c.c. of the resulting solution upon 1 c.c. of Benedict's solution. This will show the normal reducing power of the inulin solution. In case the inulin was hydrolyzed, the Benedict's test in the hydrolysis experiment should show a more pronounced reduction than that observed in the check experiment.

The color in the case of dextrin does not reappear as readily on cooling as in the case of starch.

3. To Detect Dextrin in Presence of Starch.—Treat 5 c.c. of dextrin solution with about 10 drops of starch paste. To the mixture add an equal bulk of saturated ammonium sulphate, shake vigorously, and allow to stand for five minutes. The starch is precipitated. Filter through a dry paper, and to a portion of the filtrate add a drop or two of iodine solution. The red reaction of erythro-dextrin is obtained.

4. Benedict's Test.—See if the dextrin solution will reduce Benedict's solution.

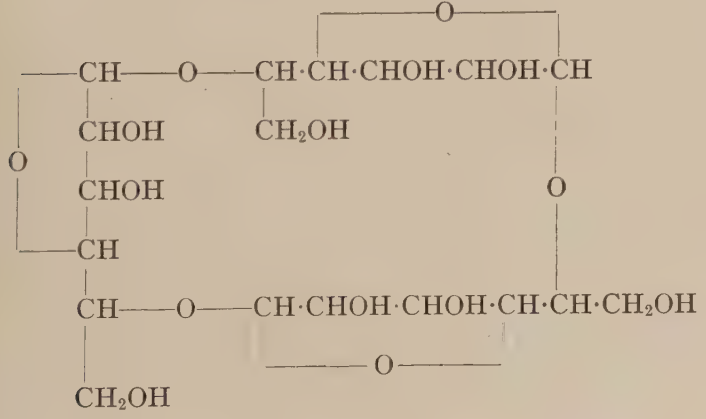
5. Hydrolysis of Dextrin.—Take 25 c.c. of dextrin solution in a small beaker, add 5 drops of dilute hydrochloric acid, and boil. By means of a small pipette, at the end of each minute, remove a drop of the solution to one of the depressions of the test-tablet and make the iodine test. The power of the solution to produce a color with iodine should rapidly disappear. When a negative reaction is obtained cool the solution and neutralize it with concentrated potassium hydroxide. Try Benedict's test (see page 76). This reaction is now strongly positive, due to the formation of a reducing sugar. Determine the nature of the sugar by means of the phenylhydrazine test (see page 72).

6. Precipitation by Alcohol.—To about 50 c.c. of 95 per cent alcohol in a small beaker add about 10 c.c. of a concentrated dextrin solution. Dextrin is thrown out of solution as a gummy white precipitate.

7. Influence of Tannic Acid.—Add an excess of tannic acid solution to a small amount of dextrin solution in a test-tube. No precipitate forms. This result differs from the result of the similar experiment upon starch (see Starch, 8, page 93).

8. Diffusibility of Dextrin.—(See Starch, 9, page 93.)

CELLULOSE, $(C_6H_{10}O_5)_x$



The above structural formula (Irvine)[1] represents a possible triglucose configuration for cellulose, but the spatial configuration and the number of units polymerized into a single molecule are not known.

[1] Irvine, and Hirst: *J. Chem. Soc.*, 123, 1518, 1923. A modification of this structural formula has been proposed by Gray, *Ind. Eng. Chem.*, 18, 811, 1926.

This complex polysaccharide forms a large portion of the cell wall of plants. It is extremely insoluble and its molecule is much more complex than the starch molecule. The best quality of filter paper and the ordinary absorbent cotton are good types of cellulose.

At one time there was but a single known solvent for cellulose. Further investigation has, however, revealed a long list of cellulose solvents. (See Experiment 7.)

Cellulose is not hydrolyzed by boiling with dilute mineral acids. It may be hydrolyzed, however, by treating with concentrated sulphuric acid then subsequently diluting the solution with water and boiling. The product of this hydrolysis is glucose.

There is some difference of opinion as to the exact extent to which cellulose is utilized in the animal organism. It is no doubt, more efficiently utilized by herbivora than by carnivora or by man. It is claimed that about 25 per cent may be utilized by herbivora, less than 5 per cent by dogs whereas the quantity utilized by man is "too small for it to play a rôle of importance in the diet of a normal individual."[1] In neither man nor the lower animals has there been demonstrated any formation of sugar or glycogen from cellulose.[2] It is probable that the cellulose which disappears from the intestine is transformed for the most part into fatty acids.[3]

Experiments on Cellulose

1. Solubility.—Test the solubility of cellulose in water, dilute and concentrated acid and alkali.

2. Iodine Test.—Add a drop of dilute iodine solution to a few shreds of cotton on a test-tablet. Cellulose differs from starch and dextrin in giving no color with iodine.

3. Formation of Amyloid.[4]—Add 10 c.c. of dilute and 5 c.c. of concentrated H_2SO_4 to some absorbent cotton in a test-tube. When entirely dissolved (without heating) pour one-half of the solution into another test-tube, cool it and dilute with water. Amyloid forms as a gummy precipitate and gives a brown or blue coloration with iodine.

After allowing the second portion of the acid solution of cotton to stand about 10 minutes, dilute it with water in a small beaker and boil for 15–30 minutes. Now cool, neutralize with solid KOH and test with Benedict's solution. Glucose has been formed from the cellulose by the action of the acid.

[1] Swartz: Transactions of the Connecticut Academy of Arts and Sciences, 16, 247, 1911.
[2] Lusk: *American Journal of Physiology*, 27, 467, 1911; also Hoffmann, Inaugural dissertation, Halle-Wittenberg, 1910.
[3] Tappeiner: *Zeitschrift für Biologie*, 24, 105 1888.
[4] This body derives its name from *amylum* (starch) and is not to be confounded with amyloid, the glycoprotein.

4. Ammoniacal Cupric Hydroxide Solubility Test (Schweitzer).—Place a little absorbent cotton in a test-tube, add Schweitzer's reagent,[1] and stir the cellulose with a glass rod. When completely dissolved acidify the solution with acetic acid. An amorphous precipitate of cellulose is produced.

5. Hydrochloric Acid Zinc Chloride Solubility Test (Cross and Bevan).[2]—Place a little absorbent cotton in a test-tube, add Cross and Bevan's reagent,[3] and stir the cellulose with a glass rod. When solution is complete reprecipitate the cellulose with 95 per cent alcohol.

6. Iodine-Zinc Chloride Reaction.—Place a little absorbent cotton or quantitative filter paper in a test-tube and treat it with the iodine-zinc chloride reagent.[4] A *blue* color forms on standing. Amyloid has been formed from the cellulose through the action of the $ZnCl_2$ and the iodine solution has stained the amyloid blue.

7. Other Cellulose Solvents.—It has been demonstrated by Deming[5] that there are many excellent solvents for cellulose (filter paper). For example, the concentrated aqueous solutions of certain salts such as *antimony trichloride*, *stannous chloride* and *zinc bromide*. In hydrochloric acid solution the solvent action of the above salts is increased. The following salts are also good solvents in hydrochloric acid solution: *mercuric chloride*, *bismuth chloride*, *antimony pentachloride*, *tin tetrachloride* and *titanium tetrachloride*. In the case of the last-mentioned salt the swollen, transparent character of the cellulose fibers preliminary to solution can be seen very nicely.

Try selected solvents suggested by the instructor.

HEMICELLULOSES

The hemicelluloses differ from cellulose in that they may be hydrolyzed upon boiling with dilute mineral acids. They differ from other polysaccharides in not being readily digested by amylases. Hemicellulose may yield pentosans, or hexosans upon hydrolysis.

Pentosans.—Pentosans yield pentoses upon hydrolysis. So far as is known they do not occur in the animal kingdom. They have, however, a very wide distribution in the vegetable kingdom, being present in leaves, roots, seeds, and stems of all forms of plants, many times in intimate association or even chemical combination with galactans. In herbivora, pentosans are 40–80 per cent utilized.[6] The few tests on

[1] Schweitzer's reagent is made by adding potassium hydroxide to a 5 per cent solution of copper sulphate, which contains 5 per cent of ammonium chloride, until precipitation is complete. A precipitate of cupric hydroxide forms and this is filtered off, washed, and 3 grams of the moist cupric hydroxide brought into solution in a liter of 20 per cent ammonium hydroxide.

[2] Cross and Bevan: *Chemical News*, 63, p. 66.

[3] Cross and Bevan's reagent may be prepared by combining two parts of concentrated hydrochloric acid and one part of zinc chloride, *by weight*.

[4] The iodine-zinc chloride reagent as suggested by Nowopokrowsky (Beihefte *Botan. Centr.*, 28, 90, 1912) may be made by dissolving 20 grams $ZnCl_2$ in 8.5 c.c. water and when cool introducing the iodine solution (3 grams KI + 1.5 gram I in 60 c.c. water) drop by drop until iodine begins to precipitate.

[5] Deming: *Journal American Chemical Society*, 33, 1515, 1911.

[6] Swartz: Transactions of the Connecticut Academy of Arts and Sciences, 16, 247, 1911.

record as to the pentosan utilization by man[1] indicate that 80–95 per cent disappear from the intestine. According to Cramer,[2] bacteria are efficient hemicellulose transformers. It has not yet been demonstrated that pentosans form glycogen in man, and for this reason they must be considered as playing an unimportant part in human nutrition. Gum arabic an important pentosan may be hydrolyzed by concentrated hydrochloric acid if boiled for a short time. The pentose arabinose results from such hydrolysis.

Galactans.—In common with the pentosans the galactans have a very wide distribution in the vegetable kingdom. The pure galactans yield galactose upon hydrolysis. One of the most important members of the galactan group is *agar-agar*, a product prepared from certain types of Asiatic sea-weed. This galactan is about 50 per cent utilizable by herbivora[3] and 8–27 per cent utilizable by man.[4] Agar ingestion has been shown to be a very efficient therapeutic aid in cases of chronic constipation.[5] This is particularly true when the constipation is due to the formation of dry, hard, fecal masses (*scybala*), a type of fecal formation which frequently follows the ingestion of a diet which is very thoroughly digested and absorbed. The agar, because of its relative indigestibility and its property of absorbing water yields a bulky fecal mass which is sufficiently soft to permit of easy evacuation. Agar has been used with good results in the treatment of constipation in children.[6] Agar is not limited to its use in connection with constipation, but may serve in other capacities as an aid to intestinal therapeutics.[7]

Experiments on a Pentosan

1. Solubility.—Test the solubility of gum arabic in hot and cold water and alcohol.

2. Iodine Test.—Add a drop of dilute iodine solution to a little gum arabic on a test-tablet. It resembles cellulose in giving no color with iodine.

3. Hydrolysis of Gum Arabic.—Introduce a little gum arabic into a test-tube, add 5–10 c.c. of strong hydrochloric acid (conc. HCl and water 1 : 1) and heat to boiling for 5–10 minutes. Cool, neutralize with potassium hydroxide and test by the Benedict or some other reduction test. A positive reaction should be obtained indicating that the gum arabic has been hydrolyzed by the acid with the

[1] König and Reinhardt: *Zeit. f. Untersuchung der Nahrungs u. Genussmittel*, 5, 110, 1902.
[2] Cramer: Inaug. Diss., Halle, 1910.
[3] Lohrisch: *Zeit. f. exper. Path. u. Pharm.*, 5, 478, 1908.
[4] Saiki: *Jour. Biol. Chem.*, 2, 251, 1906.
[5] Mendel: *Zentralblat f. d. gesammte Phys. u. Path. des Stoffw.*, No. 17, 1, 1908.
Schmidt: *Münch. med. Woch.*, 52, 1970, 1905.
[6] Morse: *Journal American Medical Ass'n.*, 55, 934, 1910.
[7] Einhorn: *Berl. klin. Woch.*, 49, 113, 1912.

production of a reducing substance. What is this reducing substance? How would you identify it?

Experiments on a Galactan

1. Solubility.—Test the solubility of agar-agar in hot and cold water. Observe its marked property of imbibing water (see above).

2. Iodine Test.—Add a drop of dilute iodine solution to a little agar-agar on a test-tablet. It resembles cellulose in giving no color with iodine.

3. Hydrolysis of Agar-agar.—Introduce a few pieces of agar-agar into a test-tube, add 5–10 c.c. of strong hydrochloric acid (conc. HCl and water 1 : 1) and heat to boiling for 5–10 minutes. Cool, neutralize with potassium hydroxide and test by the Benedict or some other reduction test. A positive reaction should be obtained indicating that the agar-agar has been hydrolyzed by the acid with the production of a reducing substance. What is this reducing substance? How would you identify it?

REVIEW OF CARBOHYDRATES

In order to facilitate the student's review of the carbohydrates, the preparation of a chart similar to the appended model is recommended. The signs + and − may be conveniently used to indicate positive and negative reaction. Only those carbohydrates which are of greatest importance from the standpoint of physiological chemistry have been included in the chart.

MODEL CHART FOR REVIEW PURPOSES

Carbohydrate	Solubility	α-Naphthol Reaction (Molisch)	Moore's Test	Benedict's Test	Nylander Test	Barfoed's Test	Iodine Test	Resorcinol-Hydrochloric Acid Reaction (Seliwanoff)	Orcinol-Hydrochloric Acid Reaction (Bial)	Mucic Acid Test	Precipitation by Alcohol	Osazone	Rotation	Diffusibility	Fermentation	Products of Hydrolysis	Remarks
Glucose.																	
Fructose.																	
Galactose																	
Pentose.																	
Maltose.																	
Lactose.																	
Sucrose.																	
Starch.																	
Inulin.																	
Glycogen.																	
Dextrin.																	
Cellulose.																	
Gum Arabic.																	
Agar-agar.																	

"Unknown" Solutions of Carbohydrates

At this point the student will be given several "unknown" solutions, each solution containing one or more of the carbohydrates studied. He will be required to detect, by means of the tests on the preceding pages, each carbohydrate constituent of the several "unknown" solutions and hand in, to the instructor, a written report of his findings, on slips furnished by the laboratory.

The scheme given on page 103 may be of use in this connection.

SCHEME FOR THE DETECTION OF CARBOHYDRATES

If the solution is alkaline, neutralize or make faintly acid with HCl. Test a few drops on a test tablet with iodine solution.

- Blue color indicates **Starch** and possibly **Dextrins.**[1]
- Reddish color indicates
  - **Erythrodextrin** if "unknown" solution is transparent after heating.
  - **Glycogen** if unknown" solution is opalescent after heating.

  If uncertain as to the differentiation between dextrin and glycogen, add ammoniacal basic lead acetate to some of the "unknown" solution.
  - No precipitate indicates **erythrodextrin.**
  - Precipitate indicates **glycogen.**
- No color with iodine solution indicates possibility of **sugars.**[2]

  Test the "unknown" solution by Benedict's test.
  - Reduction indicates **glucose, fructose, galactose, pentose, maltose, lactose** possibly **sucrose.**

    Test "unknown" with Barfoed's solution.
    - Reduction indicates **glucose, fructose, galactose, pentose,** and possibly **lactose** or **maltose.**

      Test "unknown' by Resorcinol-Hydrochloric acid (Seliwanoff). Positive test indicates **fructose.** Negative test indicates **glucose, galactose** or **pentose.**

      If Barfoed's solution is reduced, boil with excess reagent until filtrate no longer gives a reduction on further boiling. Filter and test filtrate with Benedict's solution. Reduction indicates **lactose** or **maltose.** Differentiate as above.

      Try mucic acid test on "unknown." Positive test indicates **galactose** if lactose is absent.

      Try Orcinol-Hydrochloric acid reaction (Bial), on "unknown." Positive test indicates **pentose.**

      Try osazone test on "unknown." Formation of glucosazone indicates **glucose** if fructose is absent.
    - No reduction indicates **lactose** or **maltose** or both. Differentiate by osazone reaction, mucic acid test or fermentation test.

    If Benedict s solution is reduced, boil with excess reagent until filtrate no longer gives a reduction on further boiling. Filter, acidify filtrate with conc. HCl, boil for one minute, cool, neutralize with conc. NaOH and test again with Benedict's solution. Reduction indicates the presence of **sucrose** in the "unknown" solution.
  - No reduction indicates the possible presence of **sucrose.**

    To 5 c.c. of the "unknown" solution in a test tube, add one drop of concentrated HCl and boil for one minute. Cool, neutralize with concentrated NaOH solution and test by Benedict's test.
    - Reduction indicates the presence of **sucrose** in the "unknown" solution.
    - No reduction indicates the absence of sucrose in the "unknown" solution.

[1] **Starches** may be precipitated from solution by addition of an equal volume of saturated ammonium sulphate solution containing 2 g. of iodine and 6 g. of potassium iodide in a liter of saturated solution.

[2] If iodine test indicates the presence of **polysaccharides** in the "unknown" solution, these may be precipitated from solution, before testing for the simple sugars, by the addition of three volumes of 95 per cent alcohol. Excess alcohol may be evaporated from the neutral filtrate.

CHAPTER IV

SALIVARY DIGESTION

The saliva is secreted by three pairs of glands, the submaxillary, sublingual, and parotid, reinforced by numerous small glands called buccal glands. The saliva secreted by each pair of glands possesses certain definite characteristics peculiar to itself. For instance, in man the parotid glands ordinarily secrete a thin, watery fluid, the submaxillary glands secrete a somewhat thicker fluid containing mucin, while the product of the sublingual glands has a more mucilaginous character. The saliva as collected from the mouth is the combined product of all the glands mentioned. The fact that there are pronounced variations in the composition of different fractions of saliva secreted by the same normal individual on a uniform diet has been emphasized by Lothrop and Gies.[1]

The saliva may be induced to flow by many forms of stimuli, such as *chemical*, *mechanical*, *electrical*, *thermal*, and *psychical*, the nature and amount of the secretion depending, to a limited degree, upon the particular class of stimuli employed as well as upon the character of the individual stimulus. For example, in experiments upon dogs it has been found that the mechanical stimulus afforded by dropping several pebbles into the animal's mouth caused the flow of but one or two drops of saliva, whereas the mechanical stimulus afforded by sand thrown into the mouth induced a copious flow of thin watery fluid. Again, when ice-water or snow was placed in the animal's mouth no saliva was seen, while an acid or anything possessing a bitter taste, which the dog wished to reject, caused a free flow of the thin saliva. On the other hand, when articles of food were placed in the dog's mouth the animal secreted a thicker saliva having a higher mucin content—a fluid which would lubricate the food and assist in the passage of the bolus through the esophagus. It was further found that by simply drawing the attention of the animal to any of the substances named above, results were obtained similar to those secured when the substances were actually placed in the animal's mouth. For example, when a pretense was made of throwing sand into the dog's mouth, a watery saliva was secreted, whereas food under the same conditions excited a thicker and more slimy secretion. The exhibition of dry food, in which the dog had no par-

[1]Lothrop and Gies: *Journal of the Allied (Dental) Societies*, 6, 65, 1911.

ticular interest (dry bread), caused the secretion of a large amount of watery saliva, while the presentation of moist food, which was eagerly desired by the animal, called forth a much smaller secretion, slimy in character. These experiments show it to be rather difficult to differentiate between the influence of physiological and psychical stimuli.

The amount of saliva secreted by an adult in 24 hours has been variously placed, as the result of experiment and observation, between 1000 and 1500 c.c., the exact amount depending, among other conditions, upon the character of the food.

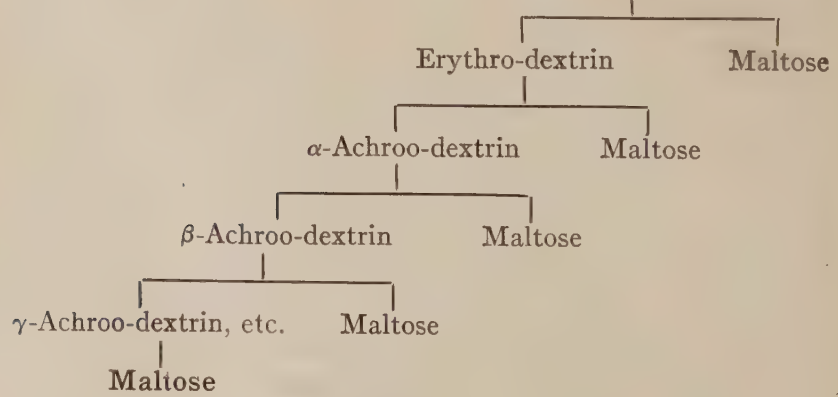
The saliva of adults ordinarily has a weak, alkaline reaction close to that of blood, but becomes acid, in some instances, 2–3 hours after a meal or during fasting, probably because of bacterial activity between the crevices of the teeth, etc. This acidity however has been shown not to be the cause of dental caries. The saliva of the newborn is generally neutral, whereas that of infants, especially those breast-fed, is generally acid.[1] The alkalinity of saliva is due principally to di-sodium hydrogen phosphate (Na_2HPO_4) and its average alkalinity may be said to be equivalent to 0.08–0.1 per cent sodium carbonate. The saliva is the most dilute of all the digestive secretions, having an average specific gravity of 1.005 and containing only 0.5 per cent of solid matter. Among the solids are found albumin, globulin, mucin, urea, the enzymes salivary amylase (ptyalin), maltase, and peptide-splitting enzymes, phosphates, and other inorganic constituents. Of the non-protein constituents of saliva, ammonia plus urea nitrogen average 76.1 per cent of that of the blood, uric acid 40.1 per cent, and total non-protein nitrogen 37.0 per cent. Normal saliva contains no glucose.[2] Potassium thiocyanate, KSCN, is also generally present in the saliva. It has been claimed that this substance is present in greatest amount in the saliva of habitual smokers. The significance of thiocyanate in the saliva is not known; it probably comes from the ingested thiocyanates and from the breaking down of protein material. The attempts to show some relationship between tooth decay and the thiocyanate content of the saliva secreted into the mouth cavity have met with failure. Certain experiments[3] indicate a virtual absence of such relationship.

The so-called tartar formation on the teeth is composed almost entirely of calcium phosphate with some calcium carbonate, mucin, epithelial cells, and organic débris derived from the food. The calcium salts are held in solution as acid salts, and are probably precipitated by the ammonia of the breath. The various organic substances just mentioned are carried down in the precipitation of the calcium salts.

[1] Allaria: *Monatsschr. für Kinderheilkunde*, 10, 179, 1911.
[2] Updegraff and Lewis, *Jour. Biol. Chem.*, 61, 633, 1924.
[3] Lothrop and Gies: *Journal of the Allied (Dental) Societies*, 6, 65, 1911

The suggestion has been made that mucin is the salivary constituent "which is particularly influential in the development of local conditions favoring the onset of dental decay."[1]

The principal enzyme of the saliva is known as *salivary amylase* or *ptyalin*. This is an *amylolytic* enzyme (see page 45), so called because it possesses the property of transforming complex carbohydrates such as starch and dextrin into simpler bodies. The action of salivary amylase is one of hydrolysis and through this action a series of simpler bodies are formed from the complex starch. The first product of the action of the ptyalin of the saliva upon starch paste is *soluble starch* (amidulin) and its formation is indicated by the disappearance of the opalescence of the starch solution. This body resembles true starch in giving a blue color with iodine. Next follows the formation, in succession, of a series of dextrins, called *erythro-dextrin*, *α-achroo-dextrin*, *β-achroo-dextrin*, and *γ-achroo-dextrin*, the *erythro-dextrin* being formed directly from *soluble starch* and later being itself transformed into *α-achroo-dextrin* from which in turn are produced *β-achroo-dextrin*, *γ-achroo-dextrin* and perhaps other dextrins. Accompanying each dextrin a small amount of maltose is formed, the quantity of maltose growing gradually larger as the process of transformation progresses. (Erythro-dextrin gives a red color with iodine, the other dextrins give no color.) The next stage is the transformation of the final dextrin into *maltose*, the latter being the principal end-product of the salivary digestion of starch. At this point a small amount of *glucose* is formed from the maltose through the action of the enzyme *maltase*. The above changes may be represented graphically as follows:[2]



[1] Lothrop and Gies : *Ibid.*, 5, No. 4, 1910.

[2] For a discussion of starch digestion see Blake: *Jour. Am. Chem. Soc.*, 38, 1245, 1916; 39, 315, 1917.

Salivary amylase acts in alkaline, neutral, or combined acid solutions. It will act in the presence of relatively strong *combined* HCl Appendix), whereas a trace (0.003 per cent to 0.0006 per cent) of ordinary *free* hydrochloric acid will not only prevent the action but will destroy the enzyme. By sufficiently increasing the alkalinity of the saliva to litmus, the action of the salivary amylase is inhibited.

It has been shown by Cannon that salivary digestion may proceed for a considerable period after the food reaches the stomach, owing to the slowness with which the contents are thoroughly mixed with the acid gastric juice and the consequent tardy destruction of the enzyme. Food in the pyloric end of the stomach is soon mixed with the gastric secretion, but food in the cardiac end is not mixed with the acid gastric juice for a considerable period of time and in this region during that time salivary digestion may proceed undisturbed. It has been shown that within 15–20 minutes after a meal 76 per cent of the starch of mashed potatoes is transformed to maltose in the normal human stomach.[1]

It has been found that salivary amylase acts more efficiently when the saliva is diluted from 4 to 7 times.[2]

Water softened by lime[3] inhibits the action of salivary amylase due to the presence of magnesium hydroxide in this water.[4] Electrolytes have an important influence upon the action of amylases. For example Rockwood[5] has shown that Cl, Br and NO_3 ions have a pronounced stimulating action upon salivary amylase.

The question of the *adaptation* of the salivary secretion to diet is one which has received considerable attention. It has been claimed, on the basis of experimental evidence,[6] that the continued feeding of a carbohydrate diet causes the secretion of a saliva which contains a higher concentration of salivary amylase and one which is therefore able more efficiently to digest the carbohydrate fed. On the other hand, strong evidence[7] has been submitted that the amylase content of the saliva is not increased through the continued feeding of a carbohydrate diet. In general the consensus of opinion is *opposed to the adaptation of digestive secretions to diet.*

[1] Bergeim: *Arch. Int. Med.*, 37, 110, 1926.
[2] Bergeim and Hawk: *Jour. Am. Chem. Soc.*, 35, 461, 1913.
[3] Prepared by treating tap water with one-sixth its volume of saturated lime water allowing to stand 24 hours and filtering.
[4] Bergeim and Hawk: *Jour. Am. Chem. Soc.*, 35, 1049, 1913.
[5] Rockwood: *Jour. Am. Chem. Soc.*, 41, 228, 1919.
[6] Neilson and Terry: *American Journal of Physiology*, 15, 406, 1905; Neilson and Lewis: *Journal of Biological Chemistry*, 4, 501, 1908.
[7] Mendel: *American Journal of the Medical Sciences*, Oct., 1909; Mendel and Underhill: *Journal of Biological Chemistry*, 3, 135, 1907; Mendel, Chapman and Blood: *Medical Record*, Aug. 27, 1910.

Maltase is the second enzyme of the saliva. The principal function of maltase is the splitting of maltose into glucose. Besides occurring in the saliva it is also present in the pancreatic and intestinal juices. For experimental purposes the enzyme is ordinarily prepared from corn. The principles of the "reversibility" of enzyme action were first demonstrated in connection with maltase by Croft Hill.

It is claimed that the saliva contains dipeptide- and tripeptide-splitting enzymes.[1] Leucyl-glycyl-alanine was the tripeptide split, whereas the cleavage of several dipeptids was brought about. The action is similar to that of intestinal erepsin (see Chapter XII). Later investigations (see page 270), apparently have demonstrated that the peptolytic power of saliva, at least in some cases, is due to bacteria.

Microscopical examination of the saliva reveals salivary corpuscles, bacteria, food débris, epithelial cells, mucus, and fungi. In certain pathological conditions of the mouth, pus cells and blood corpuscles may be found in the saliva.

Experiments on Saliva

A satisfactory method of obtaining the saliva necessary for the experiments which follow is to chew a small piece of pure paraffin wax, thus stimulating the flow of the secretion, which may be collected in a small beaker. Filtered saliva is to be used in every experiment except for the microscopical examination.

1. Microscopical Examination.—Examine a drop of unfiltered saliva microscopically, after staining with methylene blue, and compare with Fig. 34 below.

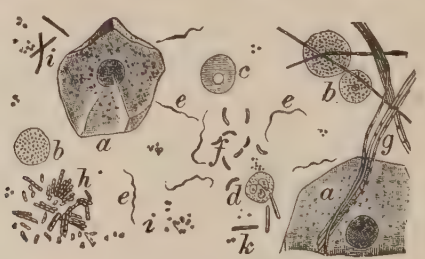


Fig. 34.—Microscopical Constituents of Saliva.
a, Epithelial cells; *b*, salivary corpuscles; *c*, fat drops; *d*, leucocytes; *e*, *f* and *g*, bacteria *h*, *i*, and *k*, fission-fungi.

2. Reaction.—Test the reaction to litmus, phenolphthalein and Congo red.

3. Specific Gravity.—Partially fill a urinometer cylinder with saliva, introduce the urinometer, and observe the reading.

4. Test for Mucin.—To a small amount of saliva in a test-tube add 1–2 drops of dilute acetic acid. Mucin is precipitated.

[1] Koelker: *Zeitschrift für physiol. Chem.*, 76, 27, 1911.

5. Biuret Test.[1]—**Render a little saliva alkaline with an equal volume of KOH and add a few drops of a very dilute (2–5 drops in a test-tube of water) copper sulphate solution. The formation of a purplish-violet color is due to mucin.**

This reaction is given by protein material and simply indicates that mucin is a protein.

6. Millon's Reaction.[2]—Add a few drops of Millon's reagent to a little saliva. A light yellow precipitate formed by the mucin gradually turns red upon being *gently* heated.

This reaction indicates the presence of protein (mucin).

7. Preparation of Mucin.—Pour 25 c.c. of saliva into 100 c.c. of 95 per cent alcohol, stirring constantly. Cover the vessel and allow the precipitate to stand at least 12 hours. Pour off the supernatant liquid, collect the precipitate on a filter and wash it, in turn, with alcohol and ether. Finally dry the precipitate, remove it from the paper and make the following tests on the mucin: (a) Test its solubility in the ordinary solvents (see page 71); (b) Millon's reaction; (c) dissolve a small amount in KOH, and try the biuret test on the solution; (d) boil the remainder, with 10–25 c.c. of water to which 5 c.c. of dilute HCl has been added, until the solution becomes brownish. Cool, render alkaline with solid KOH, and test by Benedict's solution. A reduction should take place.

Mucin is what is known as a conjugated protein or glycoprotein (see page 159) and upon boiling with the acid the carbohydrate group in the molecule has been split off from the protein portion and its presence is indicated by the reduction of Benedict's solution.

8. Inorganic Matter.—Test for chlorides, phosphates, sulphates, and calcium. For chlorides, acidify with HNO_3 and add $AgNO_3$. For phosphates, acidify with HNO_3, heat and add molybdate solution.[3] **For sulphates, acidify with HCl and add $BaCl_2$ and warm. For calcium, acidify with acetic acid, CH_3-COOH, and add ammonium oxalate, $(NH_4)_2C_2O_4$.**

9. Viscosity Test.—Place filter papers in two funnels, and to each add an equal quantity of starch paste (5 c.c.). Add a few drops of saliva to one lot of paste and an equivalent amount of water to the other. Note the progress of filtration in each case. Why does one solution filter more rapidly than the other?

10. Test for Nitrites.—Add 1–2 drops of dilute H_2SO_4 to a little saliva and thoroughly stir. Now add a few drops of a potassium iodide solution and some starch paste. Nitrous acid is formed which liberates iodine, causing the formation of the blue iodide of starch.

11. Thiocyanate Tests.—(a) Ferric Chloride Test.—To a little saliva in a small porcelain crucible, or dish, add a few drops of dilute ferric chloride and acidify slightly with HCl. Red ferric thiocyanate $Fe(SCN)_3$ forms. To show that the red coloration is not due to iron phosphate add a drop of $HgCl_2$ when colorless mercuric thiocyanate forms.

(*b*) *Solera's Reaction.*—This test depends upon the liberation of iodine through the action of thiocyanate upon iodic acid. Moisten a strip of *starch paste-iodic acid*

[1] The significance of this reaction is pointed out on p. 164.
[2] The significance of this reaction is pointed out on p. 162.
[3] See "Reagents and Solutions," Appendix.

test paper[1] with a little saliva. If thiocyanate be present the test paper will assume a blue color, due to the liberation of iodine and the subsequent formation of the so-called iodide of starch.

12. Digestion of Starch Paste.—To 25 c.c. of starch paste in a small beaker, add 5 drops of saliva and stir thoroughly. At intervals of a minute remove a drop of the solution to one of the depressions in a test-tablet and test by the iodine test. If the blue color with iodine still forms after five minutes, add another 5 drops of saliva. The opalescence of the starch solution should soon disappear, indicating the formation of soluble starch which gives a blue color with iodine. This body should soon be transformed into erythro-dextrin which gives a red color with iodine, and this in turn should pass into achroo-dextrin which gives no color with iodine. This is called the achromic point. When this point is reached test by Benedict's test to show the production of a reducing body. A positive Benedict's test may be obtained while the solution still reacts red with iodine inasmuch as some maltose is formed from the soluble starch coincidently with the formation of the erythro-dextrin. How long did it take for a complete transformation of the starch? For a graphic representation of the above changes see page 106.

13. Separation of the Products of Salivary Digestion.—To 25 c.c. of starch paste in a small beaker add 1 c.c. of saliva and stir thoroughly. At intervals of one minute test a drop of the mixture by the iodine test. If the blue color persists after five minutes add another 1 c.c. of saliva. When the mixture reacts red with iodine, indicating that erythro-dextrin has been formed, add 100 c.c. of 95 per cent alcohol. Allow to stand until the white precipitate has settled. Filter, evaporate the filtrate to dryness, dissolve the residue in 5–10 c.c. of water and try Benedict's test (page 76) and the phenylhydrazine reaction (see Glucose, 3, page 72). On the dextrin precipitate try the iodine test (page 96). Also hydrolyze the dextrin as given under Dextrin, 5, page 97.

14. Digestion of Raw Starch.—In a test-tube shake up a small amount of raw starch with a little water. Add a few drops of saliva, mix well, and allow to stand. After 10–20 minutes filter and test the filtrate by Benedict's test. What is the result?

It has been shown that *raw* corn starch and wheat starch are completely digested and absorbed by normal adults whereas *raw* potato starch is somewhat less than 80 per cent. available.[2]

15. Influence of Temperature.—In each of four tubes place about 5 c.c. of starch paste. Immerse one tube in cold water from the faucet, keep a second at room temperature, and place a third in the incubator or the water-bath at 40°C. (If the temperature of the bath or incubator is allowed to rise to 70°C. or over the enzyme is destroyed and no digestion takes place.) Now add to the contents of each of these three tubes two drops of saliva and shake well; to the contents of the fourth tube add two drops of boiled saliva. Test frequently by the iodine test, using the test-tablet, and note in which tube the most rapid digestion occurs. Explain the results.

[1] This test paper is prepared as follows: Saturate a good quality of filter paper with 0.5 per cent starch paste to which has been added sufficient iodic acid to make a 1 per cent solution of iodic acid and allow the paper to dry in the air. Cut it in strips of suitable size and preserve for use.

[2] Langworthy and Deuel: *Jour. Biol. Chem.*, 42, 27, 1920.

16. Influence of Dilution.[1]—Take a series of six test-tubes each containing 9 c.c. of water. Add 1 c.c. of saliva to tube 1 and shake thoroughly. Transfer 1 c.c. of the solution from tube 1 to tube 2 and after mixing thoroughly 1 c.c. from tube 2 to tube 3. Continue in this manner until you have transfer solutions of gradually decreasing strength. Now add starch paste in equal amounts to each tube, mix very thoroughly, and place in the incubator or water-bath at 40°C. After 10–20 minutes test by both the iodine and Benedict's tests. In how great dilution does your saliva act?

17. Influence of Acids and Alkalis.—(a) Influence of Free Acid.—Prepare a series of six tubes in each of which is placed 4 c.c. of one of the following strengths of free HCl: 0.2 per cent, 0.1 per cent, 0.05 per cent, 0.025 per cent, 0.0125 per cent and 0.006 per cent. Now add 2 c.c. of starch paste to each tube and shake them thoroughly. Complete the solutions by adding 2 c.c. of saliva to each and repeat the shaking. The total acidity of this series would be as follows: 0.1 per cent, 0.05 per cent, 0.025 per cent, 0.0125 per cent, 0.006 per cent and 0.003 per cent. Place these tubes on the water-bath at 40°C. for 10–20 minutes. Divide the contents of each tube into two parts, testing one part by the iodine test and testing the other, after neutralization, by Benedict's test. What do you find?

(b) Influence of Combined Acid (Protein Salt).—Repeat the first three experiments of the above series using combined hydrochloric acid (Appendix) instead of the free acid. How does the action of the combined acid differ from that of the free acid? (For a discussion of combined acid see page 202.)

(c) Influence of Alkali.—Repeat the first four experiments under (a) replacing the HCl by 2 per cent, 1 per cent, 0.5 per cent and 0.25 per cent Na_2CO_3. Neutralize the alkalinity before trying the iodine test (see Starch, 5, page 93).

(d) Nature of the Action of Acid and Alkali.—Place 2 c.c. of saliva and 2 c.c. of 0.2 per cent HCl in a test-tube and leave for 15 minutes. Neutralize the solution, add 4 c.c. of starch paste and place the tube in the incubator or water-bath at 40°C. In 10 minutes test by the iodine and Benedict's tests and explain the result. Repeat the experiment, replacing the 0.2 per cent HCl by 2 per cent Na_2CO_3. What do you deduce from these two experiments?

18. Influence of Metallic Salts, etc.—In each of a series of tubes place 4 c.c. of starch paste and 0.5 c.c. of one of the solutions named below. Shake well, add 0.5 c.c. of saliva to each tube, thoroughly mix, and place in the incubator or water-bath at 40°C. for 10–20 minutes. Show the progress of digestion by means of the iodine and Benedict's tests. Use the following chemicals: *Metallic salts*, 10 per cent lead acetate, 2 per cent copper sulphate, 5 per cent ferric chloride, 8 per cent mercuric chloride; *Neutral salts*, 10 per cent sodium chloride, 10 per cent magnesium sulphate, 3 per cent barium chloride, 10 per cent Rochelle salt. Also try the influence of 2 per cent carbolic acid, 95 per cent alcohol, and ether and chloroform. What are your conclusions?

Antiseptics do not necessarily inhibit enzyme action.

19. Excretion of Potassium Iodide.—Ingest a small dose of potassium iodide (0.2 gram) contained in a gelatin capsule, quickly rinse out the mouth with water, and then test the saliva at once for iodine. This test should be negative. Make additional tests for iodine at two-minute intervals. The test for iodine is

[1] The technic of Wohlgemuth's method (see Chapter XI) may be employed in this test if so desired.

made as follows: Take 1 c.c. of $NaNO_2$ and 1 c.c. of dilute H_2SO_4[1] in a test-tube, add a little saliva directly from the mouth, and a small amount of starch paste. The formation of a blue color signifies that the potassium iodide is being excreted through the salivary glands. Note the length of time elapsing between the ingestion of the potassium iodide and the appearance of the first traces of the substance in the saliva. If convenient, the urine may also be tested. The chemical reactions taking place in this experiment are indicated in the following equations:

$$(a) \qquad 2NaNO_2 + H_2SO_4 \rightarrow 2HNO_2 + Na_2SO_4.$$

$$(b) \qquad 2KI + H_2SO_4 \rightarrow 2HI + K_2SO_4.$$

$$(c) \qquad 2HNO_2 + 2HI \rightarrow I_2 + 2H_2O + 2NO.$$

[1] Instead of this mixture a few drops of HNO_3 possessing a yellowish or brownish color (containing HNO_2) may be employed.

CHAPTER V

PROTEINS:[1] THEIR DECOMPOSITION AND SYNTHESIS AMINO ACIDS

THE proteins are a class of substances which, in the light of our present knowledge, consist, *in the main*, of combinations of α-amino acids or their derivatives. These protein substances form the chief constituents of many of the fluids of the body, constitute the organic basis of animal tissue, and at the same time occupy a decidedly preeminent position among our organic food-stuffs. They are absolutely necessary to the uses of the animal organism for the continuance of life and they cannot be satisfactorily replaced in the diet of such an organism by any other dietary constituent either organic or inorganic. Such an organism may *exist* without protein food for a period of time, the length of the period varying according to the specific organism and the nature of the substitution offered for the protein portion of the diet. Such a period is, however, distinctly one of *existence* rather than one of normal life and one which is consequently not accompanied by such a full and free exercise of the various functions of the organism as would be possible upon an evenly balanced ration, *i.e.*, one containing the requisite amount of protein food. These protein substances are, furthermore, essential constituents of *all living cells* and therefore without them *vegetable life* as well as animal life is impossible.

The proteins, which constitute such an important group of substances, differ from carbohydrates and fats very decidedly in elementary composition. In addition to containing *carbon*, *hydrogen*, and *oxygen*, which are present in fats and carbohydrates, the proteins invariably contain *nitrogen* in their molecule and generally *sulphur* also. Proteins have also been described which contain *phosphorus*, *iron*, *copper*, *iodine*, *manganese*, and *zinc*. The percentage composition of the more important members of the group of protein substances would fall within the following limits: C = 50–55 per cent, H = 6–7.3 per cent, O = 19–24 per cent, N = 15–19 per cent, S = 03–2.5 per cent, P = 0.4–0.8 per cent *when present*. When *iron*, *copper*, *iodine*, *manganese*, or *zinc* are present in the protein molecule they are practically without exception present only in *traces* and with the exception of *iodine* are probably not constituents of the protein molecule.[2]

[1] The term *proteid* has been very widely used by English-speaking scientists to signify the class of substances we have called *proteins*.

[2] Some investigators regard these elements as contaminations, or constituents of some non-protein substance combined with the protein.

Of all the various elements of the protein molecule, *nitrogen* is by far the most important. The human body needs nitrogen for the continuation of life, but it cannot use the nitrogen of the air or that in various other combinations as we find it in nitrates, nitrites, etc. However, in the protein molecule the nitrogen is present in a form which is utilizable by the body. The protein molecule is made up of various nitrogen-containing components, which may be classified in the manner indicated: I, monamino acids; II, diamino acids; III, substances containing nitrogen in imid form, and IV, substances containing nitrogen as in guanidin.

The actual structure of the protein molecule is still unknown, and we have had no means by which its molecular weight could be even approximately established. Recently a method has been described for the determination of the molecular weight of proteins[1] which entails the use of an ultracentrifuge.[2] This method is based upon the measurement of sedimentation equilibrium in a protein solution exposed to a centrifugal force. The determinations by this method indicate that hemoglobin solutions are built up of molecules containing four groups of molecular weight 16,700.[3] Therefore the molecular weight of hemoglobin in aqueous solution is probably 66,800. By an entirely different procedure du Noüy[4] has shown the molecular weight of crystalline egg albumin to be 30,000. On the basis of comprehensive physico-chemical investigations Cohn, Hendry and Prentiss[5] have estimated the molecular weights of fourteen proteins as follow:

Protein	Minimal molecular weight
Gelatin	10,300
Zein	19,400
Gliadin	20,700
Hemocyanin, *Limulus*	22,700
Bence-Jones' protein	24,500
Edestin	29,000
Hemocyanin, *Octopus*	33,500
Egg albumin	33,800
Glutenin	36,300
Fibrin	42,000
Serum albumin	45,000
Hemoglobin	50,000
Serum globulin	81,000
Casein	192,000

[1] Svedberg and Fahraeus: *Jour. Am. Chem. Soc.*, 48, 430, 1926.
[2] Svedberg and Rinde: *Jour. Am. Chem.*, Soc., 46, 2677, 1924.
[3] This figure confirms the findings of other observers using different methods (Wilson: *Bioch. Jour.*, 19, 80, 1925; Barcroft and Hill: *Jour. Physiol.*, 39, 411, 1910; Hüfner and Gausser: *Arch. Anat. Physiol.*, 209, 1907.
[4] du Noüy: *Jour. Biol. Chem.*, 64, 595, 1925.
[5] Cohn, Hendry and Prentiss: *Jour. Biol. Chem.*, 63, 721, 1925. See also Cohn: *Physiological Reviews*, 5, 349, 1925.

The decomposition[1] of protein substances may be brought about by oxidation or hydrolysis, but inasmuch as the hydrolytic procedure has been productive of the more satisfactory results, that type of decomposition procedure alone is used at present. This hydrolysis of the protein molecule may be accomplished by acids, alkalis, or superheated steam, and in digestion by the action of the proteolytic enzymes. The character of the decomposition products varies according to the method utilized in tearing the molecule apart. Bearing this in mind, we may say that the decomposition products of proteins include *proteoses*, *peptones*, *peptides*, *carbon dioxide*, *ammonia*, *hydrogen sulphide*, and *amino acids*. These amino acids[2] constitute a long list of important substances which contain nuclei belonging either to the *aliphatic*, *carbocyclic*, or *heterocyclic* classes of compounds. The list includes *glycocoll* (*glycine*), *alanine*, *serine*, *phenylalanine*, *tyrosine*, *cystine*, *tryptophane*, *histidine*, *valine*, *arginine*, *leucine*, *isoleucine*, *lysine*, *aspartic acid*, *glutamic acid*, *proline*, *oxyproline*, and *norleucine*. Of these amino acids, tyrosine and phenylalanine contain carbocyclic nuclei; histidine, proline, and tryptophane contain heterocyclic nuclei; and the remaining members of the list, as given, contain aliphatic nuclei. The amino acids are preëminently the most important class of protein decomposition products. These amino acids are all α-amino acids, and, with the exception of glycocoll, are all optically active. Furthermore, they are amphoteric substances and consequently are able to form salts with both bases and acids. These properties are inherent in the NH_2 and COOH groups of the amino acids.

The decomposition products of protein may be grouped as *primary* and *secondary* decomposition products. By *primary* products are meant those which exist as radicals within the protein molecule and which are liberated, upon cleavage of this molecule, with their carbon chains intact and the position of their nitrogen unaltered. The *secondary* products are those which result from the disintegration of the primary cleavage products. No matter what method is used to decompose a given protein molecule, the primary products are largely the same under all conditions.[3]

In the process of hydrolysis the protein molecule is gradually broken down and less complicated aggregates than the original molecule are formed, which are known as *proteoses*, *peptones*, and *peptides*, and which still possess true protein characteristics. Further hydrolysis causes the

[1] The terms "degradation," "dissociation," and "cleavage," are often used in this connection.

[2] For a discussion of amino acids see Underhill's "Physiology of Amino Acids," Yale University Press, 1915.

[3] Alkaline hydrolysis yields *urea* and *ornithine* which result from *arginine*. the product of acid hydrolysis.

ultimate transformation of these substances, of a protein nature, into the amino acids of known chemical structure. In this decomposition the protein molecule is not broken down in a regular manner into ½, ¼, ⅛ portions and the amino acids formed *in a group* at the termination of the hydrolysis. On the contrary, certain amino acids are formed very early in the process, in fact while the main hydrolytic action has proceeded no further than the proteose stage. Gradually the complexity of the protein portion undergoing decomposition is simplified by the splitting off of the amino acids and finally it is so far decomposed through previous cleavages that it yields only amino acids at the succeeding cleavage. In short, the general plan of the hydrolysis of the protein molecule is similar to the hydrolysis of starch. In the case of starch there is formed a series of dextrins of gradually decreasing complexity and coincidently with the formation of each dextrin a small amount of sugar is split off and finally nothing but sugar remains. In the case of protein hydrolysis there is a series of proteins of gradually decreasing complexity produced and coincidently with the formation of each new protein substance amino acids are split off and finally the sole products remaining are amino acids.

Inasmuch as diversity in the method of decomposing a given protein does not result in an equally diversified line of decomposition products, but, on the other hand, yields products which are quite comparable in character, it may be argued that there are probably well-defined lines of cleavage in the individual protein molecule and that no matter what the force brought to bear to tear such a molecule apart, the disintegration, when it comes, will yield in every case certain definite fragments. These fragments may be called the "building stones" of the protein molecule, a term used by some of the German investigators. Take, for example, the decomposition of protein which may be brought about through the action of the enzyme *trypsin* of the pancreatic juice. When this enzyme is allowed to act upon a given protein, the latter is disintegrated in a series of definite cleavages, resulting in the formation of *proteoses*, *peptones*, and *peptides* in regular order, the peptides being the last of the decomposition products which possess protein characteristics. They are all built up from amino acids and are therefore closely related to these acids on the one side and to peptones on the other. We have *di-*, *tri-*, *tetra-*, *penta-*, *deca-*, and *poly-peptides* which are named according to the number of amino acids included in the peptide molecule. Following the peptides there are a diverse assortment of *monamino* and *diamino* acids which constitute the final products of the protein decomposition. These acids are devoid of any protein characteristics and are therefore decidedly different from the original

substance from which they were derived. From a protein of huge molecular weight, a typical colloid, perhaps but slightly soluble, and entirely non-diffusible, we have passed by way of proteoses, peptones, and peptides to a class of simpler crystalline substances which are, for the most part, readily soluble and diffusible.

These amino acids after their production in the process of digestion, as just indicated, are synthesized within the cells of the organism to form protein material which goes to build up the tissues of the body. It is thus seen that the amino acids are of prime importance in the animal economy. It was formerly believed that these essential factors in metabolism and nutrition could not be produced within the animal organism from their elements, but were only yielded upon the hydrolysis of ingested protein of animal or vegetable origin. Experiments, however, by Abderhalden and by Grafe and Schläpfer and others indicate that the nitrogen of food protein may in part be replaced by ammonium salts. Experiments by Osborne and others also indicate amino acid synthesis by animals.

Important data regarding the decomposition products of the protein molecule are given in the table on pages 118–120.

When we examine the formulas of the principal members of the crystalline end-products of protein decomposition we note that they are invariably acids, as has already been mentioned, and contain an NH_2 group in the α position. This relation of the NH_2 group to the acid radical is *constant*, no matter what other groups or radicals are present. We may have straight *chains* as in alanine and glutamic acid, the *benzene ring* as in phenylalanine, or we may have *sulphurized bodies* as in cystine and still the formula is always of the same type, *i.e.*,

$$\begin{array}{l} NH_2 \\ | \\ R—CH—COOH \end{array}$$

It is seen that this characteristic grouping in the amino acid provides each one of these ultimate fragments of the protein molecule with both a strong *acid* and a strong *basic* group. For this reason it is theoretically possible for a large number of these amino acids to combine and the resulting combinations may be very great in number, since there is such a varied assortment of the acids. The protein molecule, which is of such mammoth proportions, is probably constructed on a foundation of this sort. Many valuable data have been collected regarding the synthetic production of protein substances, the leaders in this line of investigation being Fischer and Abderhalden. After having gathered a mass of data regarding the final products of the protein decomposition

COMPARISON OF THE DECOMPOSITION PRODUCTS OF PROTAMINES, AND OTHER PROTEINS.[1]

Decomposition Product.	Protamines. (Per cent of total nitrogen of amino acid.)							Other Proteins. (Per cent of amino acids in proteins.)							
	Scombrine.	Cyclopterine.	Sturine.	α-Cyprinine	β-Cyprinine	Clupeine.	Salmine.	Gliadin.	Edestin.	Casein.	Gelatin.	Globin.	Zein.	Keratin (horse hair)	Lactalbumin[2]
Glycocoll	....	....	....	....	....	....	.....	0	3.8	0.45	25.5	0	0	4.7	0.37
Alanine	+	....	+	....	....	+	.....	2.00	3.6	1.85	8.7	4.2	13.39	1.5	2.41
Valine	....	....	....	+	+	+	1.65	3.34	6.2	7.93	1.0	....	1.88	.....	3.30
Leucine	....	....	+	....	....	....	.....	6.62	14.5	9.70	7.1	29.0	19.55	8.0	14.03
Proline	3.8	....	....	....	....	+	4.3	13.22	4.1	7.63	9.5	2.3	9.04	.....	3.76
Phenylalanine	....	....	....	....	....	....	.....	2.35	3.1	3.88	1.4	4.2	6.55	.....	1.25
Aspartic acid	....	....	....	....	....	....	.....	0.58	4.5	4.10	3.5	4.4	1.80	0.3	9.30
Glutamic acid	....	....	....	....	....	....	.....	43.66	18.74	2.77	5.8	1.7	26.17	3.7	12.89
Hydroxyglutamic acid	....	....	....	....	....	....	0.0	2.40	.....	10.50[4]	0.0	....	2.50	.....	10.0
Serine	....	....	....	....	....	+	3.25	0.13	0.33	0.50	0.4	0.6	1.02	.....	1.76
Tyrosine	....	2.2	....	+	1.5	....	.....	3.50	2.1	6.50	0.01	1.3	3.55	3.2	1.95
Arginine	88.8	67.7	63.5	8.7	28.0	88.0	89.2	3.14	14.2	3.81	8.2	7.9	1.82	.....	3.47[3]
Lysine	....	....	8.4	30.3	6.6	.....	.....	0.92	3.7	7.62	5.9	11.1	0	.....	9.87[3]
Histidine	....	....	11.8	....	....	....	.....	3.35	3.92	2.84	0.9	13.0	0.82	.....	2.61[3]
Tryptophane	+	+	....	....	....	....	.....	1.14	2.48	2.20	0	+	0	+	+
Cystine	....	....	....	....	....	....	.....	2.32	1.00	0.50	0.31	0.3	0.85	11.0	1.73[3]
Hydroxyproline	....	....	....	....	....	....	.....	?	0	0.23	14.1	1.0	?	.....	.....
Ammonia	....	....	....	....	....	....	.....	5.22	2.3	1.61	0.49	5.4	3.64	.....	1.31[3]

and demonstrating that amino acids were the ultimate results of the various forms of decomposition, these investigators, and notably Fischer, set about in an effort to form, from these amino acids, by synthetic means, substances which should possess protein characteristics. The simplest of these bodies formed in this way was synthesized from two molecules of glycocoll with the liberation of water, thus:

$$H_2N-CH_2-CO\ OH\ H\ HN-CH_2-COOH.$$

[1] Kossel: *Zeit. physiol. Chem.*, 44, 347, 1905.
Osborne and Guest: *Jour. Biol. Chem.*, 9, 425, 1911.
Abderhalden, Kossel and others.
Abderhalden, Fischer, Mörner, Dakin and others.
Fischer, Levene and Aders: *Zeit. physiol. Chem.*, 35, 70, 1902; also Levene and Beatty: *Ibid.*, 49, 252, 1906. Dakin: *Jour. Biol. Chem.*, 44, 499, 1920.
Abderhalden: *Zeit. physiol. Chem.*, 37, 484, 1903.
Osborne and Liddle, *Am. Jour. Physiol.*, 26, 295, 1910.

[2] Jones and Johns: *Jour. Biol. Chem.*, 48, 347, 1921.

[3] Osborne, Van Slyke, Leavenworth and Vinograd: *Jour. Biol. Chem.*, 22, 259, 1915.

[4] Dakin: *Bioch. Jour.*, 12, 290, 1918.

AMINO ACIDS OBTAINED UPON DECOMPOSITION OF THE PROTEIN MOLECULE

Nomenclature.	Nucleus.[1]	Formula.	Class.	Optical activity.	Discovered.		Maximum yield.			
					By	Date.	Per cent.	Obtained by	Date.	From decomposition of
Arginine: α-amino-δ-guanidin-valeric acid.	A	$C_6H_{14}N_4O_2$..	Monobasic, diamino.	*d*	Schulze and Steiger.	1886	89.20[2]	Kossel and Pringle.	1906	Salmine.
Alanine: α-amino-propionic acid.	A	$C_3H_7NO_2$...	Monobasic, monamino.	*d*	Schützenberger and Bourgeois.	1875	24.00	Abderhalden and Spack.	1909	Tussah silk fibroin.
Lysine: α-ε-diamino-caproic acid.	A	$C_6H_{14}N_2O_2$..	Monobasic, diamino.	*d*	Drechsel.....	1889	28.80	Kossel and Dakin.	1904	α-Cyprinine.
Histidine: α-amino-β-imidazolyl-propionic acid.	C	$C_6H_9N_3O_2$..	Monobasic, monamino.	*l*	Kossel.....	1896	12.90	Kossel and Kutscher.	1900	Sturine.
Proline: α-pyrrolidine-carboxylic acid.	H. P.	$C_5H_9NO_2$...	Pyrrol derivative.	*l*	Fischer.....	1901	13.73	Osborne and Clapp.	1907	Hordein.
Tryptophane: α-amino-β-indole-propionic acid.	H. I.	$C_{11}H_{12}N_2O_2$.	Indol derivative.	*l*	Hopkins and Cole.	1901	1.5(?)	Fischer.....	1901	Casein.
Hydroxyproline: Hydroxy-α-pyrrolidine carboxyilc acid	H. P.	$C_5H_9NO_3$...	Pyrrol derivative.	*l*	Fischer.....	1902	14.1	Dakin.....	1920	Gelatin.
Isoleucine: α-amino-β-methyl-β-ethyl-propionic acid.	A	$C_6H_{13}NO_2$..	Monobasic, monamino.	*d*	Ehrlich.....	1903	3.0	Levene, Van Slyke and Birchard.	1910	Fibrin-Heteroproteose.
Norleucine (*Caprine*): α-amino caporic acid.	A	$C_6H_{13}NO_2$	Monobasic, monamino.	*dl*	Abderhalden and Weil	1913		Abderhalden and Weil	1913	Nerve tissue

[1] Aliphatic; C = Carbocyclic; H. I. = Hetercyclic (Indole ring); H. P. = Heterocyclic (Pyrrol ring). [2] Per cent of total nitrogen.

AMINO ACIDS OBTAINED UPON DECOMPOSITION OF THE PROTEIN MOLECULE.—*Continued*

Nomenclature.	Nucleus.[1]	Formula.	Class.	Optical activity.	Discovered.		Maximum yield.			
					By	Date.	Per cent.	Obtained by	Date.	From decomposition of
Cystine: di (β-thio-α-amino-propionic acid).	A	$C_6H_{12}O_4N_2S_2$	(Disulphide of) monobasic, monamino.	*l*	Wollaston.....	1810	13.92	Mörner.....	1901	Human hair.
Leucine: α-amino-iso-butyl-acetic acid.	A	$C_6H_{13}NO_2$...	Monobasic, monamino.	*l*	Proust.....	1818	29.04	Abderhalden	1903	Globin.
Glycocoll: amino acetic acid.	A	$C_2H_5NO_2$....	Monobasic, monamino.	*i*	Braconnot.....	1820	37.5	Abderhalden and Behrend.	1909	Canton silk.
Aspartic acid: amino-succinic acid.	A	$C_4H_7NO_4$....	Dibasic, monamino.	*l*	Plisson.....	1827	23.80	Abderhalden and Strauss	1906	Spongin.
Tyrosine: α-amino-β-para-hydroxyphenyl-propionic acid.	C	$C_9H_{11}NO_3$...	Monobasic, monamino.	*l*	Liebig.....	1846	10.06	Kutscher....	1903	Zein.
Valine: α-amino-iso-valeric acid.	A	$C_5H_{11}NO_2$...	Monobasic, monamino.	*d*	Gorup-Besanez	1856	7.20	Osborne and Guest.	1911	Casein.
Serine: α-amino-β-hydroxy-propionic acid.	A	$C_3H_7NO_3$....	Oxymonamino	*l*	Cramer.....	1865	7.80	Kossel and Dakin.	1904	Salmine.
Glutamic acid: α-amino-normal-glutaric acid.	A	$C_5H_9NO_4$....	Dibasic, monamino.	*d*	Ritthausen....	1866	43.66	Osborne and Guest.	1911	Gliadin.
Hydroxyglutamic acid: α-amino-β-hydroxy-glutaric acid.	A	$C_5H_9NO_5$....	Dibasic, monamino.	*d*	Dakin.....	1918	10.5	Dakin	1918	Casein.
Phenylalanine: β-phenyl-α-amino-propionic acid.	C	$C_9H_{11}NO_2$...	Monobasic, monamino.	*l*	Schulze and Barbieri.	1881	6.96	Langstein...	1903	Zein.

[1] A = Aliphatic; C = Carbocyclic; H. I. = Heterocylic (Indole ring); H. P. = Heterocyclic (Pyrrol ring).

The body thus formed is a *dipeptide*, called *glycyl-glycine*. In an analogous manner may be produced *leucyl-leucine*, through the synthesis of two molecules of leucine or *leucyl-alanyl-glycine* through the union of one molecule of leucine, one of alanine, and one of glycocoll. By this procedure Fischer and his pupils have been able to make a large number of peptides containing varied numbers of amino acid radicals, the name *polypeptides* being given to the whole group of synthetic substances thus formed. One of the most complex polypeptides yet produced is one containing fifteen glycocoll and three leucine residues.

Notwithstanding the fact that most synthetic polypeptides are produced through a union of amino acids by means of their imide bonds, it must not be imagined that the protein molecule is constructed from amino acids linked together in straight chains in a manner analogous to the formation of simple peptides, such as glycyl-glycine. The molecular structure of the proteins is much too complex to be explained upon any such simple formation as that. There must be a variety of linkings, since there is a varied assortment of decomposition products of totally different structure.

Many of these synthetic bodies respond to the biuret test, are precipitated by phosphotungstic acid, and behave, in other ways, as to leave no doubt as to their protein characteristics. For instance, a number of amino acids each possessing a *sweet* taste have been synthesized in such a manner as to yield a polypeptide of *bitter* taste, a well-known characteristic of peptones. From the fact that the polypeptides formed in the manner indicated have free acidic and basic radicals we gather the explanation of the amphoteric character of true proteins.

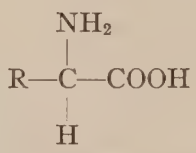
AMINO ACIDS

The proteins may be hydrolysed in a number of ways: by the action of acids, or of alkalis, or of the appropriate enzymes, or even by superheated steam. Where a more or less quantitative separation of the hydrolytic products is desired, concentrated hydrochloric acid or 10 per cent sulphuric acid is usually employed. The isolation of the amino acids depends upon processes involving one or more of the following: fractional precipitation, fractional crystallization, fractional distillation and extraction.

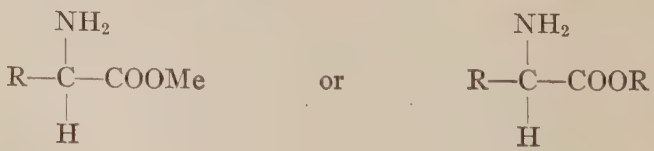
Fractional precipitation is employed in the precipitation of tryptophane by mercuric sulphate, of histidine by mercuric chloride and of the "hexone bases" (arginine, lysine and histidine) by phosphotungstic acid. Fractional crystallization is illustrated in the recovery of leucine, cystine, tyrosine and glutamic acid from solutions, the process

being based on relative insolubilities. Emil Fischer employed the principle of fractional distillation with remarkably successful results. The amino acids of the mixture were first converted into their corresponding esters (by dissolving them in absolute alcohol and saturating with dry hydrogen chloride) and these were then distilled under constantly diminishing pressure and constantly increasing temperature. The fourth method, the method of direct extraction, was introduced by Dakin when he showed that butyl alcohol extracts monamino acids only, leaving behind the dicarboxylic as well as the diamino acids.

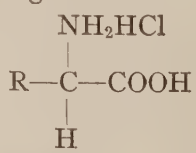
Physical Properties.—The amino acids are usually well-defined crystalline substances with high, and rather indefinite melting points. They are fairly soluble in cold, and readily soluble in hot water. They are soluble in acid and alkaline solutions, forming salts in either case, and insoluble in ether. Glycocoll, the simplest of the group, is sweet to the taste, and so are some of the others; but a number of them have either no taste at all, or, like the proteins, are bitter. With the exception of glycocoll, they are all optically active, possessing one or more asymmetric carbon atoms:



Chemical Properties.—The amino acids, with 5 exceptions, are amphoteric in character, due to the combined influence of the NH_2 and COOH groups. Lysine, histidine and arginine are basic, for they contain more than one NH_2 group, whereas glutamic and aspartic acids are acidic, since they contain more than one COOH group. Containing as they do both the NH_2 and COOH groups, two types of salts are possible. With metallic hydroxides or alcohols we get



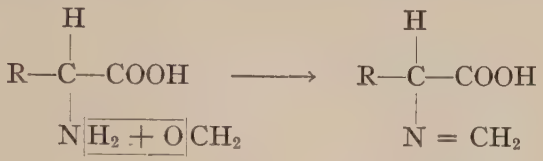
and with acids (*e.g.* HCl) we get



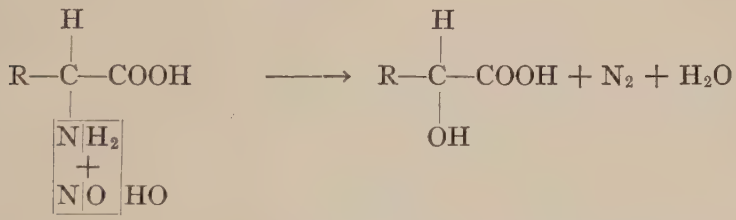
Due to the presence of the NH_2 group, the amino acids form "addition" compounds with a number of "neutral" salts, such as

sodium chloride, lithium chloride, calcium chloride, copper chloride, mercuric nitrate, mercuric chloride and bismuth chloride. These "addition" compounds, the chemistry of which is not altogether clear, are sometimes of value in separating and purifying the amino acids.

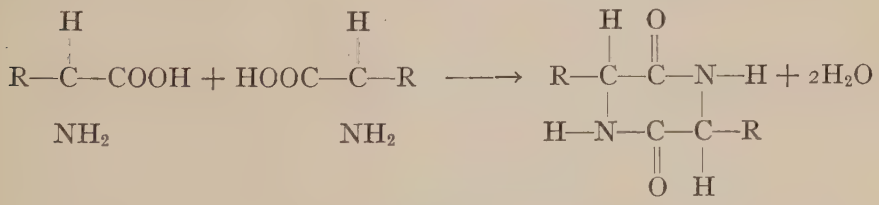
With aldehydes the amino acids form methylene derivatives:



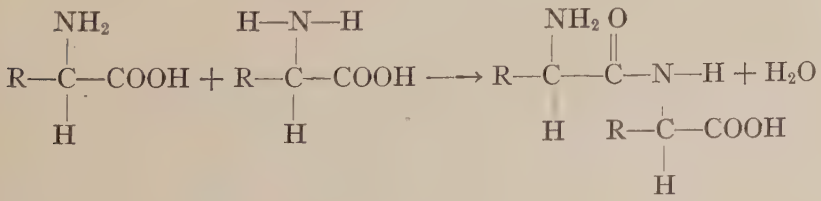
in the course of which the basic character of the amino group is destroyed. This reaction forms the basis for the method employed by Sørensen in estimating amino acids. With nitrous acid the corresponding hydroxy acids are obtained:



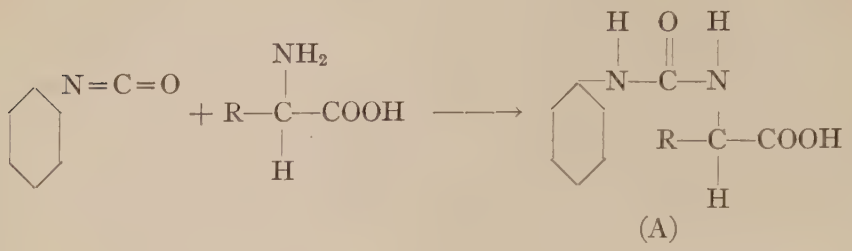
and this reaction has been used by Van Slyke to determine amino acids. Very often, by the mere evaporation of a water solution of an amino acid, two of its molecules will combine to form diketopiperazine derivatives:



A reaction of far-reaching importance in the attempted synthesis of proteins depends upon the property these amino acids possess of combining with one another to form peptids:

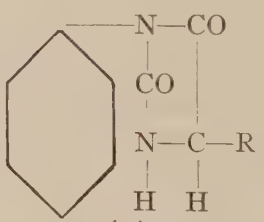


The amino acids combine with phenyl isocyanate to form phenyl isocyanate compounds of amino acids (A)



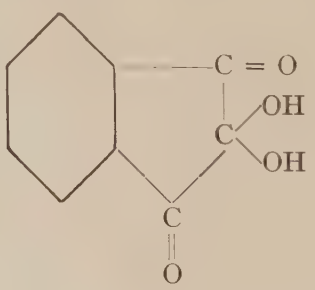
(A)

which, in turn, by treatment with hydrochloric acid, can be converted into the corresponding hydantoin derivatives (B):



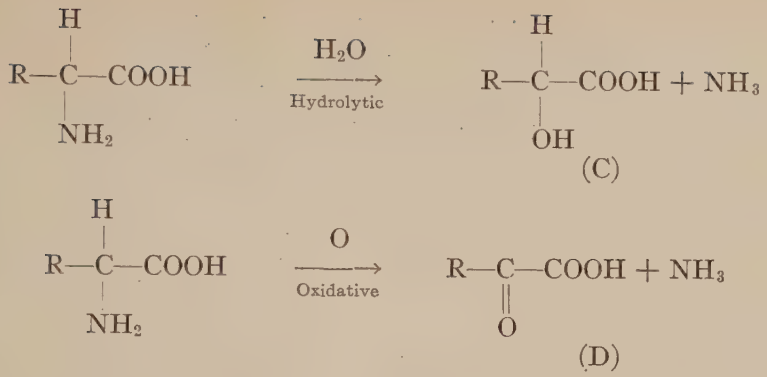
(B)

With ninhydrin (triketohydrindene hydrate),



upon heating, the amino acids develop a blue color. Only α-amino acids give this reaction, but it is also given by proteins and their various hydrolytic products.

Physiological Properties.—As a rule, the amino acids are deaminized in the body into the corresponding hydroxy or keto acids, the ammonia which is given off combining for the most part with carbon dioxide to form, ultimately, urea, which is eliminated:



The concensus of opinion now favors the oxidative rather than the hydrolytic process, though it is still a matter of debate as to just where this reaction takes place. The fact that the amino acids are carried *via* the portal circulation to the liver would argue in favor of this organ as the seat for the chemical transformation; and that this is true has been shown experimentally.

Not all of the amino acids undergo this transformation for some are needed by the tissues for the repair or for the direct synthesis of tissue proteins. There is no appreciable storage within the body either of amino acids or of their more complex relatives, the proteins.

The hydroxy or keto compound (C or D) may next undergo one or both of two changes: it may either start on a series of chemical transformations the end product of which is glucose or it may be transformed into β-hydroxybutyric acid, which is an intermediary product in the metabolism of fats. Glycocoll, alanine, serine, cysteine, aspartic and glutamic acids are among the glucose-formers, while valine, leucine, isoleucine, phenylalanine and tyrosine give rise to β-hydroxybutyric acid. Histidine is somewhat of an exception. It is probable that its imidazol group is excreted either as uric acid or allantoin, while its side chain (alanine) forms urea.[1] Tryptophane is also an exception: Dakin has shown that this substance does not form either glucose or β-hydroxybutyric acid in the organism of the dog.

Claude Bernard believed that glucose could arise from protein. It has long been known that "extra sugar" appears in the urine of persons suffering from diabetes mellitus; also the amount of sugar is to a large degree proportional to the amount of protein ingested. As there is considerable difference in the amino acid content of different proteins, this observation was quite to be expected. It remained for Graham Lusk and his coworkers to make a detailed study of the sugar-forming capacity of the individual amino acids. The experimental method was in general conducted according to the following plan: In order to

[1] Harrow and Sherwin: Unpublished data.

prove conclusively the origin of sugar from amino acids they first digested meat artificially with pancreatic extract and fed this mixture of amino acids to a dog which had been rendered diabetic by the use of phlorhizin. They found that the nitrogen was quickly eliminated in the urine, and for each gram of such nitrogen appearing in the urine 3.5 grams of extra glucose were excreted. Nitrogen makes up only about 16 per cent of the protein molecule, the other 84 per cent consisting chiefly of carbon and hydrogen. Hence for excretion of one gram of nitrogen in the urine we may calculate that approximately 6.25 grams (*i.e.* $^{100}/_{16}$) of protein have been destroyed. Finally then if the destruction of 6.25 gms. of protein causes the formation of 3.65 gms. of glucose, we may conclude that 58.4 per cent of protein is convertible into glucose.

The formation of β-hydroxybutyric acid has been studied in the cases of individual amino acids on diabetic persons, on animals rendered diabetic or by perfusing these amino acids in the blood through the surviving liver and then making determinations of β-hydroxybutyric acid in the blood leaving this organ.

The nutritional value of the different amino acids varies considerably. Osborne and Mendel, for example, have shown that when zein, a protein present in maize, becomes the sole source of the protein supply offered to rats, the animals fail to grow and develop normally. An analysis of zein reveals the fact that it is deficient in the amino acids lysine and tryptophane. The addition of these amino acids to zein provides the impetus for growth and normal development. Using such methods of testing, it was found that at least four amino acids are absolutely essential for normal development: lysine, tryptophane, cystine and tyrosine. One can go even further and say that life without them is impossible. Histidine has been added to this list of essential amino acids by Rose[1] and proline may also be required.[2] At an early date it was found that casein, the chief protein of milk, contains very little glycocoll. Since milk is a most important dietary factor in the early life of all young mammals it was concluded that the animal body has the power to synthesize glycocoll. As a proof of this, it is a well known fact that the animal or human being fed benzoic acid will detoxicate this and by combining it with glycocoll will excrete the hippuric acid thus formed in the urine. The fact that the body is able to accomplish this synthesis when glycocooll is not stored in the body or available in the food makes it quite apparent that the appearance of the glycocoll conjugate in the urine must be a result of a direct synthesis. In this same way the *synthesis* of other "unessential"

[1] Rose, W. and Cox: *Jour. Biol. Chem.*, 61, 747, 1924.
[2] Sure: *Jour. Biol. Chem.*, 64, 325, 1925.

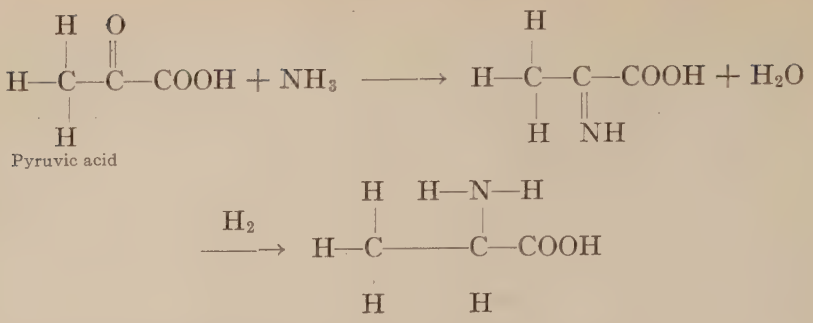
amino acids in the body can be proved. If phenylacetic acid is fed to an animal it is combined with glycocoll, but if ingested by a human being, it is excreted in the urine in combination with glutamine. Thus a man, placed on a carbohydrate diet, ingested first benzoic acid and it was found that the glycocoll for the detoxication of the acid was synthesized, without the destruction of body tissue, and the nitrogen for the synthesis was taken from the waste fraction or that nitrogen which would otherwise have appeared in the urine in the form of urea. After the ingestion of phenylacetic acid on the same protein-free diet it was found that the body would synthesize glutamine in the same way from the waste or urea nitrogen. When both benzoic acid and phenylacetic acid were fed simultaneously the body synthesized both glycocoll and glutamine, excreting phenyl acetyl glutamine and hippuric acid in the urine, at the same time reducing the urea nitrogen from 65 to 12 per cent of the total N but without perceptibly increasing the total nitrogen output.[1]

A hen fed benzoic acid combines this acid with ornithine, an essential part of the amino acid arginine. If the hen is fed benzoic acid and kept on a diet of rice she will synthesize the ornithine for the detoxication[2] of the benzoic acid not at the expense of the urea fraction of the urine but from the uric acid nitrogen thus decreasing the output of uric acid, as it is a well known fact that fowls excrete the major part of their waste nitrogen as uric acid and not as urea. This would tend to show that the unessential amino acids can probably all be synthesized in the body. It is of interest to know whether the "essential amino acids" as classified by the nutritional chemist can be synthesized under like conditions.

If a dog is fed brombenzene on a meat diet, he will excrete the brombenzene in combination with cysteine. If the proteins of the diet be withdrawn, thereby cutting off the exogenous supply of cysteine, he will excrete the brombenzene as an ethereal sulphate or glycuronate.[3] Thus it is shown that the *essential amino acid cysteine* can not be synthesized in the animal body even though various forms of sulphur and nitrogen be furnished with the diet.[4]

It has been suggested by Knoop that perhaps there is a condensation of ammonia with a ketonic acid to form the amino acid *in vivo*. This reaction would be the reverse of the reaction in which amino acids are converted into ketonic acids and would likely pass through the *imino* stage as follows:

[1] Shiple and Sherwin: *Jour. Amer. Chem. Soc.*, 44, 618, 1922.
[2] Crowdle and Sherwin: *Jour. Biol. Chem.*, 55, 365, 1923.
[3] Shiple, Muldoon and Sherwin: *Jour. Biol. Chem.*, 60, 59, 1924.
[4] Muldoon, Shiple and Sherwin: *Jour. Biol. Chem.*, 59, 675, 1924.



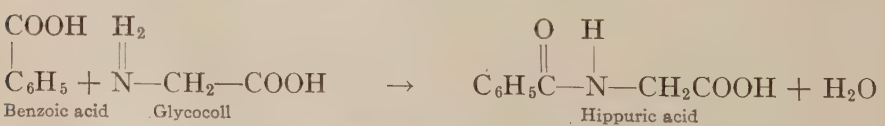
Unfortunately it has been shown[1] that lysine cannot be formed from its corresponding alpha keto acid.

Discussion of the Individual Amino Acids

Glycocoll (Glycine).—Glycocoll is prepared synthetically by the action of ammonia on monochloracetic acid.[2] It crystallizes in rather large colorless monoclinic crystals usually in 4 sided prisms. It is soluble in water and saturated ammonium sulphate solution but insoluble in alcohol and ether. Its water solution possesses a sweet taste. It melts with decomposition at 232–236°. Glycocoll possesses no asymmetric carbon atom and is therefore not optically active. For crystals see Fig. 35, p. 130.

Both the carbon atoms of glycocoll are converted into glucose in the phlorhizinized dog. This amino acid is not an essential one, and is absent from the protein of some very important foods, such as milk, for example. The nitrogen of glycocoll is, so far as we know, entirely converted into urea.

Glycocoll is no doubt most important on account of its wide adaptability by the detoxicating mechanism of the body and is of especial interest to the student of intermediary metabolism. More than a hundred substances have been found which when fed are joined in the body with glycocoll. When glycocoll is employed by the body as a detoxicating agent it is joined only with an organic acid in true dipeptide style. In other words, the amino group of glycocoll interacts with the carboxyl group of the toxic compound "splitting out water" and joining the two compounds in the following manner:



[1] Lewis, McGinty and Marvel: *Jour. Biol. Chem.*, 59, xiii, 1924.

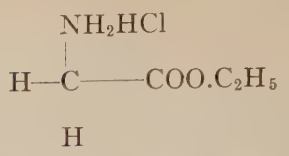
[2] Keenan: *Jour. Biol. Chem.*, 62, 165, 1924. Drushel and Knapp: *Chem. Zent.*, 1, 144, 1916.

If 5 or 6 gms. of benzoic acid are ingested the acid is almost quantitatively combined with glycocoll during the first 12 hours and excreted in the urine as hippuric acid. The same process takes place when the subject is fasting or on a protein free diet. As glycocoll is not stored in the body and an exogenous supply has been cut off, there remains but one possibility, *i.e.* that glycocoll is synthesized in the animal body. This synthesis apparently takes place at the expense of the waste material in the body, since the amount of nitrogen found in the synthesized glycocoll is taken from the urea fraction of the urine, there being a corresponding decrease in the urea nitrogen for the same period. The nitrogen obtained from the "extra protein" does not result from body tissue destruction as suggested by Ringer[1] for there is no increase in total urinary nitrogen excepting after large doses of benzoic acid. Large single doses cause destruction of considerable body tissue but only a small proportionate increase of glycocoll,[2] but even this is not employed quantitatively in the work of detoxication. Glycocoll is found in bile combined with cholic acid and called glycocholic acid.

(1) **Preparation of Glycocoll from Hippuric Acid. Put 5 gms of hippuric acid in a 100 c.c. round bottom flask. Attach the flask to a reflux condenser after adding 20–25 c.c. of 30 per cent sulphuric acid. Now boil the contents of the flask on a wire gauze for one hour, cool under the tap and filter. The boiling acid splits the hippuric acid into benzoic acid and glycocoll. The benzoic acid being quite insoluble in cold water now begins to crystallize out and is retained on the paper. In order to remove the remainder of the benzoic acid from the filtrate, shake the filtrate with twice its volume of ether in a separatory funnel. The filtrate is next diluted with three volumes of water and heated with sufficient barium carbonate until the sulphuric acid has been precipitated out as barium sulphate. Filter off the barium sulphate and evaporate the filtrate on a water bath until the glycocoll begins to crystallize out.**

(2) **Preparation from Glue.—Place 300 c.c. concentrated HCl and 100 gms. of ordinary glue in a 600 c.c. round bottom flask. Heat on a water bath with frequent shaking until the glue has gone into solution. Connect the flask with a reflux condenser, place on wire gauze and heat to boiling for 3–4 hours. Transfer contents of flask to a liter distilling flask and distill in vacuo, until the contents of the flask become thick and viscous. Attach the flask again to the reflux condenser, pour 500 c.c. absolute alcohol into the flask and heat until the solution in the alcohol is complete. Add to the alcoholic solution 5 gms. animal charcoal and boil for 1–2 minutes. Filter hot, cool the filtrate under the water tap and saturate with dry HCl gas. Boil under reflux condenser on a water bath for one hour, cool and allow the flask to stand over night in the ice box. The glycocoll ethyl ester hydrochloride should crystallize out as a mass of colorless needles. In case this does not occur, it may be necessary to "concentrate in vacuo." The glycocoll ethyl ester hydrochloride may be represented by the following formula.**

[1] Ringer: *Jour. Biol. Chem.*, 10, 327, 1911.
[2] Lewis: *Jour. Biol. Chem.*, 18, 225, 1924.

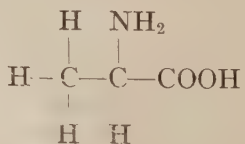


(3) Preparation of Free Glycocoll.—Weigh the glycocoll ethyl ester hydrochloride, place in a 250 c.c. round bottomed flask and add 2 c.c. of water for each gram of the substance. Next add about 100 c.c. ether, cool in a freezing mixture and introduce 30 per cent NaOH until the aqueous layer is neutral to litmus. (0.8–1 gm. is required for every gram of the hydrochloride.) Next add potassium carbonate (powdered) with vigorous shaking, until the aqueous layer becomes pasty. The alkali splits off the hydrocholic acid leaving the glycocoll ethyl ester which is soluble in ether. Pour off the ether. Dry the

Fig. 35.—Glycocoll (Synthetic).
(From Keenan, *Jour. Biol. Chem.*, 62, 163, 1924.). See also Fig. 83.

ether by shaking for 5–10 minutes with 20 gms. of anhydrous potassium carbonate. Decant the ether solution and remove the last traces of water by treatment with anhydrous sodium sulphate. For this it is best to place the ether in a tightly stoppered bottle or flask with about 25 gms. of the anhydrous sodium sulphate. Filter. Distill off the ether under reduced pressure the receiver being kept cool with ice. Change the receiver. Distill off glycocoll ester at 44° and 11 mm. pressure, keeping the receiver cool. To the distillate in a round bottom flask add 10 volumes of water. Boil with reflux condenser until no longer alkaline. Evaporate in a porcelain dish on the water bath. Glycocoll should crystallize out. For crystalline form see Fig. 35.

d-**Alanine,** $C_3H_7NO_2$ is *α-amino propionic acid* and is represented as follows:



It is best prepared from silk, in which it occurs to the extent of 21 per cent, according to the method of Fischer.[1] Alanine is easily soluble in hot water and quite insoluble in cold water, alcohol and acetone. It has a sweetish taste, is dextrorotatory and melts with decomposition between 293–297°. For crystals of alanine see Fig. 36.

Alanine does not appear to be an "essential amino acid" or a necessary constituent of the proteins of our diet. There is also no proof at the present time that it is actually synthesized in the animal body although this has been suggested by Knoop. This amino acid is a complete sugar-former. In other words, all three carbon atoms are

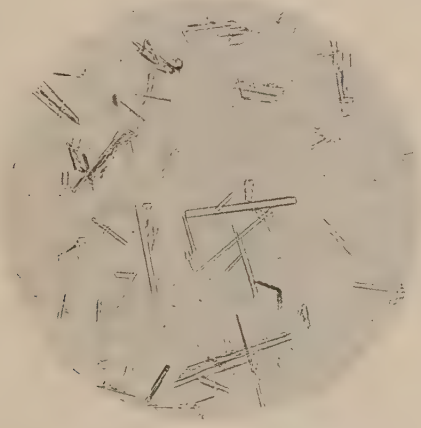
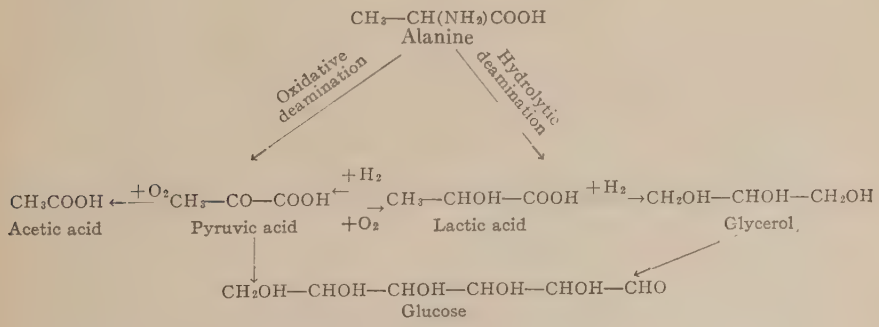


FIG. 36.—ALANINE. (From Keenan, *Jour. Biol. Chem.*, 62, 163, 1924.)

capable of forming glucose in the diabetic organism. Alanine probably occupies a very important position in the processes of intermediary metabolism. Through deamination the acid is converted into either lactic or pyruvic acid, which compounds are important "go-betweens" of proteins and carbohydrates as well as more indirectly of the fats.

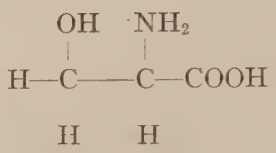


[1] Fischer and Skita: *Zeit. physiol. Chem.*, 33, 177, 1909.

The above scheme illustrates this relationship of alanine to the carbohydrate glucose and to glycerol, a part of the fat molecule.

Certain resistant ring structures such as benzene and indol can not be oxidized in these forms by the body cells but combined with alanine they can be very readily oxidized in the processes of normal metabolism. Such combinations form some of our most important amino acids, as phenylalanine, histidine, tyrosine and tryptophane.

l-**Serine,** *α-amino-β-hydroxy propionic acid,* has the following structural formula:



The acid was prepared synthetically from glycol aldehyde, hydrocyanic acid and ammonia by Fischer and Leuchs.[1] It was first obtained by

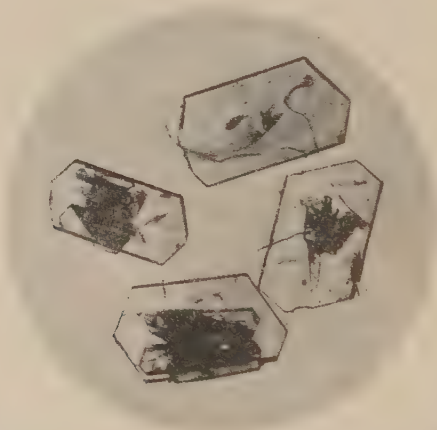
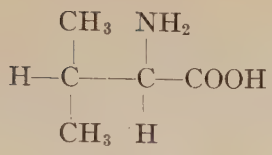


FIG. 37.—SERINE. (From Keenan, *Jour. Biol. Chem.*, 62, 163, 1924.)

Cremer in fairly large quantities from the hydrolysate of silk. It crystallizes from water solution as thin irregular plates, has a sweet taste and usually is inactive when isolated from a protein hydrolysate although as it exists in protein it is believed to be laevorotatory. It melts at about 245° and is soluble in 23 times its weight of water at room temp. Serine, like alanine, is completely (all 3 carbon atoms) convertible into sugar and apparently forms no acetoacetic acid in the animal organism. For crystals of serine see Fig. 37.

d-**Valine,** *α-amino-isovaleric acid,* $C_5H_{11}NO_2$, has the following structural formula:

[1] Fischer and Leuchs: *Ber.*, 35, 3787, 1902.



It is made synthetically by the interaction of ammonia on α-brom-isovaleric-acid under pressure in which process the bromine is split off as HBr and the amino group takes the α-position originally occupied by the bromine.

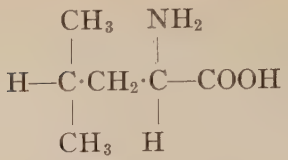
Valine occurs in casein to the extent of 7.20 per cent whereas edestin contains 6.20 per cent. It has not been demonstrated to be an essential amino acid from the dietary standpoint. Little is known concerning the intermediary metabolism of valine. It does not appear to be converted into glucose in the phlorizinized animal[1] nor does it appear to produce any appreciable amount of acetoacetic acid[2] in the course of its metabolism. In fact the intermediary metabolism of the "branched chain" aliphatic acids is still in doubt but it would seem that the side chain is perhaps one of the first points of attack after the amino group, thus convering it into a "straight chain" compound.

Leucine, *α-amino isobutyl acetic acid*, $C_6H_{13}NO_2$, is found in nearly all proteins.

Normally it is present in pancreas, thymus, thyroid, spleen, brain, liver, kidney and salivary glands. It has been found *pathologically* in urine (in acute yellow atrophy of the liver, in acute phosphorus poisoning and acute febrile conditions).

The laevo compound is the one most commonly isolated from hydrolyzed protein. The pure compound is only slightly soluble in cold water or absolute alcohol but is quite soluble in hot dilute alcohol. It melts at 295–297°C. with decomposition. Pure leucine crystallizes in shiny, white, extremely thin crystals. The inactive leucine tastes slightly sweet, the *d*-leucine quite sweet and *l*-leucine slightly bitter. Leucine is apparently not an essential amino acid from the dietary standpoint. It is not convertible into glucose in the animal body[3] but forms considerable β-hydroxybutyric acid when fed to the diabetic.[4] It is apparently first deaminized then oxidized in the alpha position thereby being converted into isovaleric acid, which then undergoes β-oxidation and is converted into β-hydroxybutyric acid. It has the following structure:

[1] Dakin, *Jour. Biol. Chem.*, 14, 321, 1913.
[2] Emden, Solomon and Schmidt: *Hofmeister's Beiträge*, 8, 129, 1906.
[3] Halsey: *Amer. Jour. Physiol.*, 10, 229, 1904.
[4] Baer and Blum: *Arch. Exp. Path. and Pharm.*, 55, 89, 1906.



Preparation of Leucine.—In a two liter flask, place 1 liter of defibrinated blood and gradually add 150 c.c. of concentrated sulphuric acid shaking well during the additions. Heat in a boiling water bath for 12–15 hours, shaking well at frequent intervals. Next boil on a sand bath for 12–14 hours, being careful to shake continually until it boils evenly. To the hot liquid add a solution of barium hydroxide until the mixture is alkaline to litmus. Filter on a Buchner funnel. Make the filtrate acid to litmus with dilute H_2SO_4, concentrate in a porcelain dish over an open flame to 500 c.c. and filter again. Make

FIG. 38.—LEUCINE.

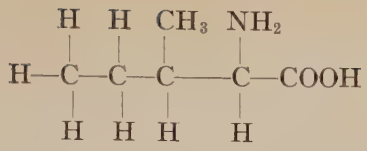
For the material from which the above crystals were prepared, as well as those reproduced in Figs. 39 to 47 inclusive the authors are indebted to Dr. Thomas B. Osborne of New Haven, Conn.

the filtrate faintly alkaline to litmus through the addition of ammonia and concentrate on a boiling water bath until a crystalline mass forms on top of the liquid. Cool for 24 hours in the ice box. Filter on a Buchner and press the water out of the crystals. Recrystallize from 70 per cent alcohol. For crystals see Fig. 38.

Experiments on Leucine.—Make the following tests upon the leucine crystals already prepared or upon some pure leucine furnished by the instructor.

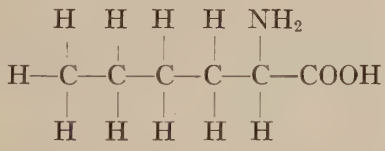
1, 2 and 3. Repeat these experiments according to the directions given under Tyrosine (page 150).

d-**Isoleucine** is $C_6H_{13}NO_2$ (*α-amino-β-methyl-β-ethyl propionic acid*) and possesses the following graphical formula:



This acid was first prepared by Ehrlich[1] from the refuse material in the sugar industry and later he prepared it synthetically[2] by the action of malonic acid ester on secondary butyl iodide. Isoleucine has a bitter taste. It crystallizes in plates like leucine. These melt at 280° and are soluble in 26 parts of water, very soluble in hot glacial acetic acid and hot methyl alcohol. This acid probably has no particularly important role in the physiology of nutrition. It has not been shown to be either an essential amino acid or to be synthesized in the animal organism. It yields no sugar in the diabetic organism and it is still a question whether it will produce β-hydroxybutyric acid. On fermentation it yields according to Ehrlich *d*-amyl alcohol.

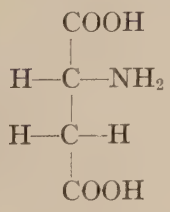
Nor-leucine, ($C_6H_{13}NO_2$) *normal α-amino caproic acid,* is represented graphically as follows:



It was much more recently discovered that its two isomers, being first isolated from protein by Abderhalden and Weil[3] in 1912.

This compound has much the same properties as isoleucine and leucine. It is quite insoluble in water and most organic solvents. It has a slightly sweetish taste, melts at about 280°, but is unlike leucine or isoleucine in regards to its intermediary metabolism, being a sugar former and not a producer of acetone bodies.

l-**Aspartic acid** ($C_4H_7NO_4$), *α-amino-succinic acid,* has the following structural formula:



This acid unlike the other acids thus far considered is strongly acidic on account of the predominance of the carboxyl group. The chief source

[1] Ehrlich: *Ber.*, 37, 1809, 1904.
[2] Ehrlich: *Ber.*, 41, 1453, 1908.
[3] Abderhalden and Weil, *Zeit. physiol. Chem.*, 84, 39, 1913.

of this acid is the monamide, asparagin, which is very widely distributed in the vegetable world and particularly abundant in the asparagus plant. Aspartic acid crystallizes in rhombic prisms, has a strong acid taste, but is rather insoluble in water (1 part in 256 parts water at 10° and in 18 parts of water at 100°). In the diabetic organism aspartic acid forms glucose. It has been found three of the four carbon atoms enter into glucose. Lusk[1] believes that the probable transformation is first through a conversion into malic acid, secondly into lactic acid and finally into glucose. It is not believed to be an essential amino acid for growth. It is more than likely that the amide (asparagin) rather than aspartic acid occurs in the protein molecule, but is quickly hydrolyzed

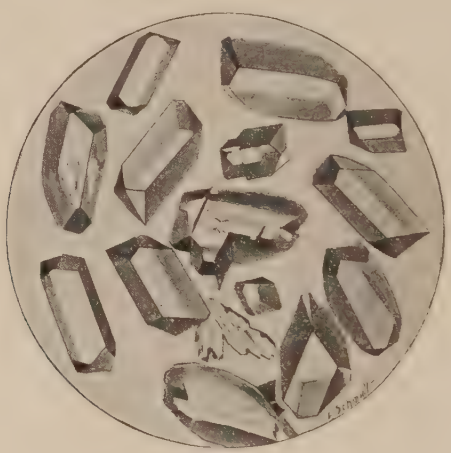
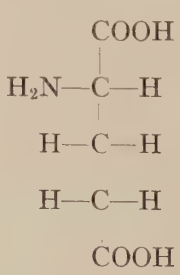


FIG. 39.—ASPARTIC ACID.

into aspartic acid and ammonia during the process of protein cleavage. For crystals of aspartic acid see Fig. 39.

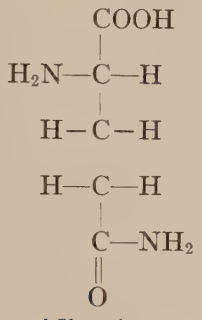
d-**Glutamic acid,** *α-amino glutaric acid*, ($C_5H_9NO_4$) may be represented structurally as follows:



Glutamic acid, the product obtained after the hydrolysis of the protein molecule, is without doubt a secondary product. The primary constit-

[1] Ringer and Lusk: *Zeit. physiol. Chem.*, 66, 106, 1910.

uent of the protein molecule is undoubtedly *d*-glutamine, the amide of *d*-glutamic acid, since it has been found by various investigators[1] that the percentages of glutamic acid and ammonia are practically always parallel in the amount of their yield after the hydrolysis of the protein molecule. Again it was shown by Thierfelder and Sherwin[2] that phenylacetic acid fed to a human being is detoxicated by conjugation with glutamine and excreted in the urine as phenylacetyl-glutamine.



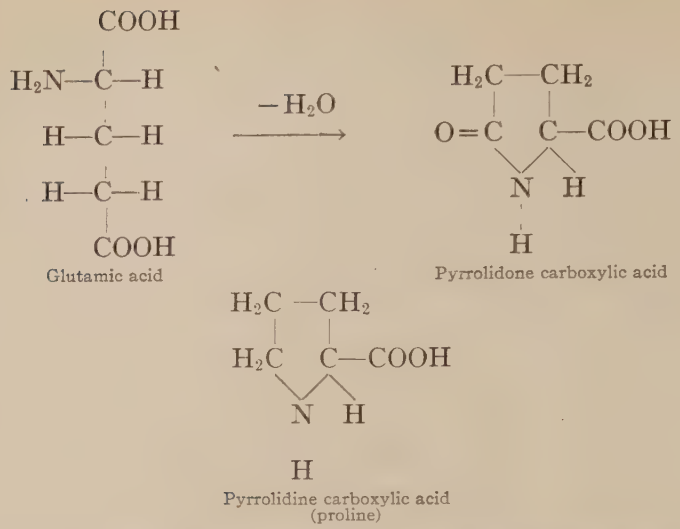
d-Glutamine

d-glutamic acid is present in practically all proteins and has been found as a larger percentage component of a single protein than any other amino acid, being present to the extent of 44 per cent in gliadin, a protein found in wheat. It also occurs to the extent of 22 per cent in muscle.

It crystallizes from water in rhombic tetrahedra which melt from 209–213°. It is readily soluble in water, but somewhat less in alcohol. It possesses two carboxyl groups and but one amino group and is therefore strongly acid toward litmus. The addition of HCl to the acid forms the hydrochloride which is extremely soluble in acid, but relatively insoluble in absolute alcohol. This property is usually employed in the isolation of glutamic acid. Glutamic acid is partially converted into glucose. Three carbon atoms are used in the sugar synthesis probably through β-oxidation of the central carbon atoms. This acid is not essential to growth although it is universally found as a component of protein. In the form of glutamine it can be synthesized, like glycocoll, on a non-protein diet from the nitrogen which would have otherwise been excreted as urea. It is synthesized only in the human body (not in other animals) after the ingestion of phenylacetic acid.

If glutamic acid is heated to approximately 190°, it forms a ring structure (pyrrolidone carboxylic acid) which is structurally allied to proline, thus:

[1] Osborne and Jones: *Amer. Jour. Physiology*, 26, 305, 1910.
[2] Thierfelder and Sherwin: *Ber.*, 47, 2630, 1914.



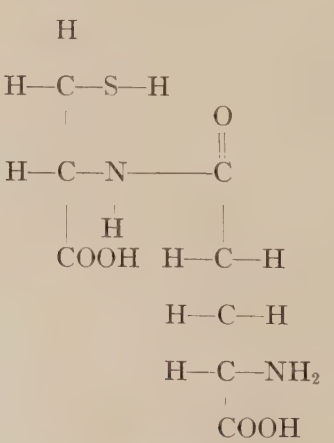
Glutamic acid

Pyrrolidone carboxylic acid

Pyrrolidine carboxylic acid (proline)

It has been suggested by various authors that pyrrolidone carboxylic acid probably marks the transition stage from proline to glutamic acid. This transition is however most improbable since proline, probably an essential amino acid for growth can neither be replaced by glutamic acid nor by pyrrolidone carboxylic acid.

A much more important role of glutamic acid is its relation to the glutathione molecule of Hopkins, the structure of which has recently been shown by Stewart to be a dipeptide combination of cystein and glutamic acid:



Glutathione

The oxidation and reduction brought about by this dipeptide is thought to be due to the alternate closing of the —SH radicals:

$$R—SH + HS—R \longrightarrow R—S—S—R + H_2.$$

Glutamic acid is not believed to be concerned with either oxidation or reduction. The function of the dipeptide may well explain the large amount of glutamic acid present in muscle as well as in many other proteins.

Preparation of Glutamic Acid.[1]—In a 500 c.c. flask place 100 gms. of gluten flour and 300. c.c. concentrated HCl. Heat on a water bath until the gluten has dissolved. Add 15–20 gms. of charcoal, then boil under a reflux condenser for 6 hrs. on a wire gauze. Cool, dilute with an equal volume of water and filter. Evaporate to about one-fourth the volume in vacuo. Transfer the residue to a 250

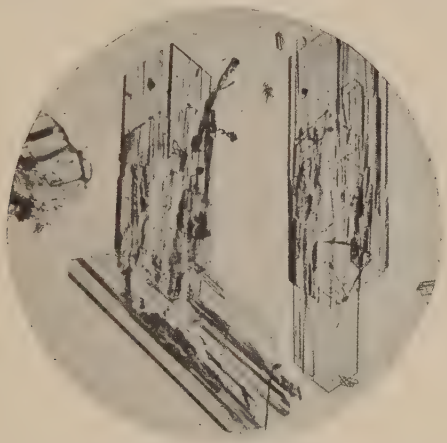


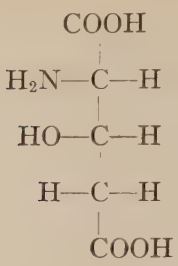
FIG. 40.—GLUTAMIC ACID.
Reproduced from a micro-photograph made by Prof. E. T. Reichert, of the University of Pennsylvania.

c.c. Erlenmeyer flask and saturate with dry HCl gas, then place in the ice box for 2–3 days. By this time crystals of glutamic acid hydrochloride should have formed. Add an equal volume of ice cold alcohol and filter through a Buchner funnel. Instead of recrystallizing the crystals, wash in the Buchner with ice-cold concentrated HCl.

In order to prepare the free glutamic acid from the glutamic acid hydrochloride, dissolve the crystals in the least amount of warm water (40–50°) then add normal alkali until the solution no longer gives a blue coloration with Congo red. Evaporate the solution to 60 c.c. in vacuo at 40°. Allow this solution to remain in the ice box until crystallization is complete. Filter off the crystals on a Buchner funnel, wash with a little cold water and dry. Test the melting point of the crystals. Examine microscopically, see Fig. 40.

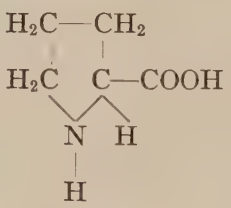
d-**Hydroxyglutamic acid** (*α-amino-β-hydroxyglutaric acid*) has the following structural formula:

[1] Schmidt and Foster (*Proc. Soc. Expt. Biol. and Med.*, 18, 205, 1921) have shown that Ajinomoto (a commercial preparation of Na-glutamate) is a good source of glutamic acid.



This was first obtained by Dakin[1] from hydrolyzed casein and later by Jones and Johns[2] from lactalbumin. It is dextrorotatory. It crystallizes from water slowly in short thick prisms. It is quite soluble in water and glacial acetic acid, slightly soluble in methyl alcohol, practically insoluble in alcohol, ether and ethyl acetate. It melts at 140-150°.

l-**Proline,** $C_5H_9NO_2$ *α-pyrrolidine-carboxylic acid,* has the following graphic structure:



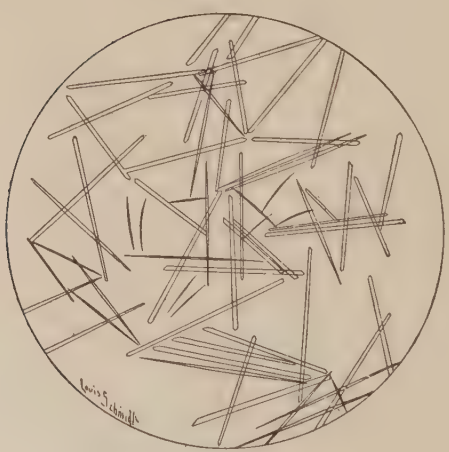


FIG. 41.—LEVO-α-PROLINE.

It was first obtained from casein, but has since been found in most proteins excepting the protamines. It is one of the few heterocyclic amino acids found in protein. Sørensen synthesized the compound

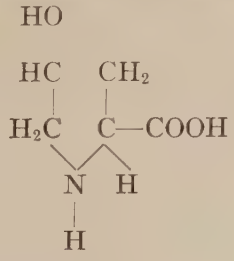
[1] Dakin: *Biochem. Jour.*, 12, 290, 1918.
[2] Jones and Johns: *Jour. Biol. Chem.*, 48, 347, 1921.

from α-amino-δ-oxy-valeric acid. Proline is easily soluble in alcohol and in cold water. It has a sweet taste and melts at 153–154°. Proline in the light of recent researches has assumed a much more important role in biological chemistry. It contains the pyrrolidine ring and therefore may be closely related to the hemoglobin of blood and the chlorophyll of plants, since both are believed to contain a complex of such rings. The assumption, however, that proline may be synthesized in the body at the expense of glutamic acid with pyrrolidone carboxylic acid as an intermediary product is groundless. In fact the contrary view has received strong support in the results obtained by Sure[1] which seem to indicate that proline is an essential amino acid for the promotion of

FIG. 42.—COPPER SALT OF PROLINE.
Reproduced from a micro-photograph made by Prof. E. T. Reichert, of the University of Pennsylvania.

growth in the animal organism. Little is known regarding the metabolism of proline excepting that Dakin[2] has shown that three of the five carbon atoms may be converted into glucose in the phlorhizinized dog. For crystalline form of proline and its copper salt see Figs. 41 and 42.

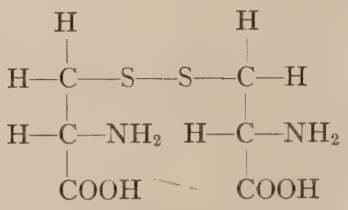
l-**Hydroxyproline,** ($C_5H_9NO_3$), *γ-hydroxy-α-pyrrolidine carboxylic acid*, may be represented graphically by the following structure:



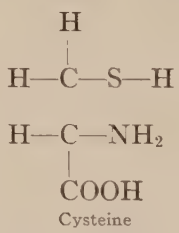
[1] Sure: *Jour. Biol. Chem.*, 59, xvi, 1924.
[2] Dakin: *Jour. Biol. Chem.*, 13, 513, 1913.

This acid was first discovered by Fischer[1] in the hydrolysate of gelatine. It is very difficult to separate from the other alpha-amino acids. It is, however, easily soluble in water and difficultly soluble in absolute alcohol. The general metabolism of hydroxyproline is probably the same as that of proline.

Cystine, ($C_6H_{12}N_2S_2O_4$). Friedmann has shown cystine to be *di-β-thio-α-amino-propionic acid* and to possess the following structural formula:



Cystine is the principal if not the only sulphur containing body resulting from the hydrolysis of simple protein.[2] It is obtained in the greatest amount as a decomposition product of keratin-containing tissue such as horn, hoof, feather and hair. Cystine crystallizes in characteristic hexagonal plates which are only very slightly soluble in cold water and in alcohol. It dissolves readily in mineral acids or in alkalis but it is insoluble in acetic acid. Upon reduction cystine is converted into two molecules of cysteine which, however, in the air or in alkaline solution oxidize readily to cystine.



Cysteine

Sullivan[3] has described a method for distinguishing between the two compounds. Cysteine is joined with glutamic acid in the structure of glutathione (see Glutamic Acid p. 136). Cysteine is employed by the dog for the detoxication of brombenzene. It is interesting to note, however, that cysteine is only excreted in combination with brombenzene when protein containing cystine is fed, thus showing that cystine or cysteine cannot be synthesized by the animal body[4] as is the case for glycocoll and glutamine.[5] This would be expected however since

[1] Fischer: *Ber.*, 35, 2660, 1902.

[2] Mueller (*Jour. Biol. Chem.*, 56, 157, 1923; 58, 373, 1923) claims to have isolated a new sulphur-containing amino acid of unknown structure.

[3] Sullivan: *Jour. Biol. Chem.*, 59, 1, 1924.

[4] Muldoon, Shiple and Sherwin: *Jour. Biol. Chem.*, 59, 675, 1924.

[5] Thierfelder and Sherwin: *Zeit. physiol. Chem.*, 94, 1, 1915.

nutritional experiments have repeatedly shown that cystine is a necessary component of the protein of the diet if growth and health are to be maintained.

Lusk[1] believes that all six carbon atoms of cystine may enter into the formation of sugar in the diabetic organism. This probably happens through an early conversion into cysteine and then perhaps into serine.

The intermediary catabolism of cystine is rather complicated as the molecule appears "open to oxidation" through the conventional NH_2 group and also through the S-H group. Experiments have shown that the first point of attack is through the NH_2 group.[2] At least there are two paths which cystine may follow during its catabolism—the one involves oxidative deamination with the conversion of the NH_2 into ammonia and urea, while the S of the molecule is largely oxidized and excreted as inorganic sulphate. A second pathway is through taurine, which is a normal constituent of the bile and apparently a cysteine oxidation product. The "ethereal sulphates"[3] found in the urine are apparently for the most part derived from endogenous catabolism and not from ingested cystine.[4]

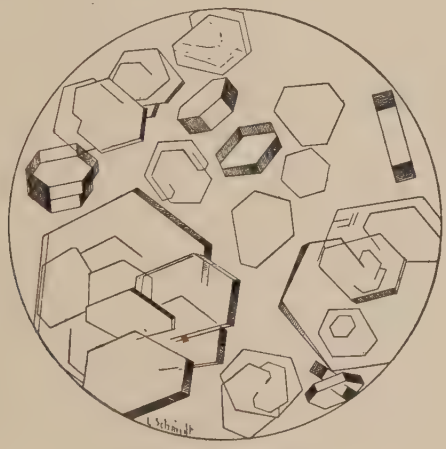


FIG. 43.—CYSTINE.

Preparation of Cystine by Folin's Method.[5]—**Take 200–250 grams of hair (barber shop sweepings) in a 2.5 liter flask, pour in 500 c.c. of ether and boil under a reflux condenser on a water bath for a few minutes in order to dissolve the oil and fat on the hair. Pour off the ether, add to the contents of the flask 500 c.c. conc. HCl, boil under a reflux condenser on a sand bath for 5–6 hours or until the mixture no longer yields a biuret test. To the hot mixture add solid**

[1] Lusk: Science of Nutrition, Third Edition, page 198.
[2] Rose, Shiple and Sherwin: *Amer. Jour. Physiol.*, 60, 518, 1924
[3] Shiple, Muldoon and Sherwin: *Jour. Biol. Chem.*, 60, 59, 1924.
[4] Muldoon, Shiple and Sherwin: *Proc. Soc. Exp. Biol. and Med.*, 20, 46, 1922.
[5] Folin, *Jour. Biol. Chem.* 8, 9, 1910.

sodium acetate to remove the free HCl. (In order to determine when the free HCl has been removed, test with Congo red paper. When the paper no longer shows a blue color the HCl has been removed.) Allow the mixture to stand 12–24 hours. Filter in a Buchner. Redissolve the precipitate in 100 c.c. 20 per cent HCl and boil with about 5 gms. of animal charcoal until clear. Filter. *Wash the charcoal well.* Again add a hot conc. solution of sodium acetate until the free HCl has been removed. Allow to stand in an ice box until crystallization is complete. Filter on a Buchner funnel, wash with cold water, then alcohol and allow to dry in air. Examine microscopically, see Fig. 43.

Preparation of Cystine by Schmidt's Method.[1]—"Human hair or wool which has been freed from oil by extraction with gasoline is hydrolyzed by heating at 100°C. with twice its weight of concentrated HCl. It requires about 12 hours to effect complete hydrolysis. The mixture must not be heated for any length of time beyond the point at which the biuret test is either negative or feebly positive since, as Van Slyke[2] has shown, cystine is destroyed during the process of hydrolyzing the protein. The greater part of the protein is removed by distilling in vacuo at a temperature between 60–70°C. and the original volume of the solution is restored by the addition of water. A thick aqueous suspension of commercial finishing lime is now slowly added, care being taken to avoid any considerable rise in temperature, until the mixture has acquired a chocolate color. It is then filtered by suction through a Buchner funnel and the residue washed a number of times with distilled water. The filtrate should be clear and possess a light brown color. Hydrochloric acid is now added to partially neutralize the alkaline solution and it is finally acidified by addition of acetic acid. On standing over night in the ice chest, sedimentation of the crude cystine takes place. This is filtered off and is dissolved in a minimum quantity of 5 per cent HCl. The solution is decolorized by boiling for several minutes with a small quantity of charcoal which has been previously boiled with HCl to remove the calcium phosphate, and the cystine is precipitated by the addition of sodium acetate to the hot solution until a drop of the solution ceases to turn Congo red paper blue. The mixture is filtered at once and the cystine is washed a number of times with hot water to completely remove the last traces of tyrosine. Typical hexagonal plates of cystine are obtained." (See Fig. 43.)

Sherman Biological Test for Cystine.[3]—This method is intended to estimate the amount of cystine in a protein (in this case casein) by comparing the growth curves of white rats fed 6 weeks on a basal diet insufficient in cystine with the growth curves of another set of rats fed the same diet plus a known amount of pure cystine (or of known amounts of casein or other protein whose cystine content is to be determined). The authors emphasize the necessity of using large numbers of experimental animals whose nutritional characteristics, etc. are known to be constant. They found that essentially equal gains in body weight were made by the rats whether the diet contained 1 per cent casein or 0.025 per cent cystine, indicating that casein contains 2.5 per cent cystine; although with less perfectly comparable experiments they got figures as low as 1.3–1.5 per cent cystine in casein. The technic of this test is described in Chapter XXXII.

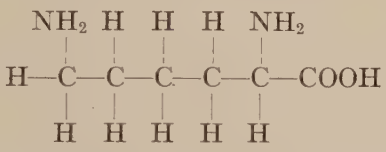
[1] Schmidt: *Proc. Soc. Exp. Biol. Med.*, 19, 50, 1921.
[2] Van Slyke: *J. Biol. Chem.*, 10, 38, 1911.
[3] Sherman and Woods: *Jour. Biol. Chem.*, 66, 29, 1925.

Qualitative Tests for Cysteine. (a) **Sullivan's Test.**[1]—**Take one or two c.c. of a solution suspected of containing cysteine in a test tube, add 1–2 drops of a 0.5 per cent solution of 1-2-naphthoquinone-4-sodium sulphonate and then 5 c.c. of 20 per cent sodium thiosulphate made up in 0.25 normal NaOH. If cysteine is present a brilliant red persists while cystine gives no color. This is a test for a "free S-H group."**

(b) **Test cysteine solution also with a dilute solution of $FeCl_3$. An indigo blue color appears and disappears almost immediately. Add a dilute solution of $CuSO_4$, whereupon a transitory violet color appears.**

(c) **Test one or two c.c. of cysteine solution with a dilute solution of sodium nitroprusside and a drop of NaOH. A deep purple violet color appears but gradually fades after a few minutes.**

Lysine, $C_6H_{14}N_2O_2$ *α-ε-diamino-caproic acid*, has the following structural formula:



It is one of the three basic amino acids possessing a predominance of amino groups over acidic or carboxyl groups. The other two amino acids belonging to this group, histidine and arginine, with lysine, make up the triad known as hexone bases.

Lysine is present in most proteins of animal origin. It is notably absent in zein and present in rather small amounts in gliadin. It has not been prepared thus far in a pure crystalline state since it decomposes easily during the process of isolation.

Lysine is absolutely essential for growth and cannot be synthesized in the body as has been shown by the classical experiments of Osborne and Mendel.[2] These experimenters fed young rats on a diet with a protein content limited to gliadin. The rats failed to grow or mature and it was presumed that lysine was the missing factor as lysine is not present in the protein gliadin. This supposition was verified by the addition of lysine to the diet, thereby causing the rats to resume growth and mature normally. (See Chapter XXXII.) It has been shown[3] that it is even impossible for the animal organisms to convert α-hydroxy-ε-amino caproic acid into lysine. In this case it would merely be necessary to bring about the interaction of ammonia with the hydroxyl group, thus forming water and replacing the hydroxyl group with the amino group. Lysine is the only straight chain amino acid which does

[1] This test has been modified for quantitative purposes and also to include cystine (Sullivan: *Public Health Reports*, 41, May 28, 1926).

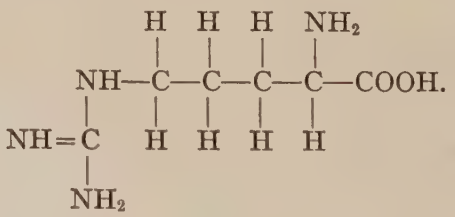
[2] Osborne and Mendel: *Jour. Biol. Chem.*, 25, 1, 1916, etc.

[3] McGinty, Lewis, and Marvel: *J. Biol. Chem.*, 62, 75, 1924.

not form glucose. Dakin[1] fed lysine to a phlorhizinized dog but found neither extra sugar nor an increase in β-hydroxy butyric acid excretion in the urine. It has been suggested that as lysine exerts such a powerful influence in such small quantities it may have something to do with the internal secretions. Its intermediary catabolism is, at least up to the present time (September, 1926), shrouded in mystery. For crystals of lysine picrate see Fig. 44.

FIG. 44.—LYSINE PICRATE.

Arginine, $C_6H_{14}N_4O_2$ is *α-amino δ-guanidine valeric acid*, and possesses the following chemical structure:



Arginine is present in most proteins and the difficulty attending its isolation from the hydrolysate of such amino acid mixtures has made experimental work with arginine-free diets almost impossible. Active arginine crystallizes in rosette-like formations. It reacts strongly alkaline, decomposes at 205°–207° and is easily soluble in water but almost insoluble in alcohol.

Arginine is acted upon by an enzyme (*arginase* of the liver) and split into urea and ornithine (*α-δ-diamino valeric acid*). This is perhaps one of the minor sources of urea formed in the body.

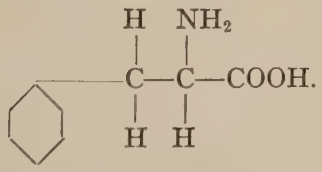
[1] Dakin: *Jour. Biol. Chem.*, 14, 321, 1913.

Repeated attempts to prove the genesis of guanidine from arginine have met with failure. It is possible that in certain disturbances of metabolism guanidine may be split off and in this way account for some physiological disturbances. When guanidine or its salts are introduced into the blood stream of an animal fibrillary contractions of the muscles and a high arterial tension may result. For the possible relationship of guanidine to parathyroid tetany see Chapter XX on Endocrine Organs.

Three carbon atoms of arginine seem to be converted into glucose in the diabetic organism. Arginine is probably an essential amino acid since it cannot be synthesized in the organism.

FIG. 45.—PHENYLALANINE.

Phenylalanine, $C_9H_{11}NO_2$. This compound is *α-amino-β-phenyl propionic acid* and is represented graphically as follows:



It is fairly insoluble in water, crystallizing from warm concentrated solutions in small shiny leaves, but from dilute solutions in fine needles. It melts with decomposition between 275°–280°. This acid is easily soluble in hot water, quite insoluble in cold water, and but little soluble in ethyl and methyl alcohol. It has a slightly bitter taste. For crystals see Fig. 45.

In the diabetic organism phenylalanine yields no sugar but is apparently capable of transformation into β-hydroxybutyric acid. The benzene ring which is practically impervious to oxidation in the body is

in this case easily oxidized to $CO_2 + H_2O$. It was formerly believed that oxidation took place in the para position simultaneously with the removal of the NH_2 group of the chain, but it is suggested by Shambaugh and Lewis[1] that in the oxidation of the aromatic group of phenylalanine a *diphenol* is formed as an intermediary product and that tyrosine and phenylalanine do not follow the same course in intermediary metabolism. Phenylalanine is perhaps to a large degree responsible for the positive xanthoproteic reaction (see p. 163) given by many proteins.

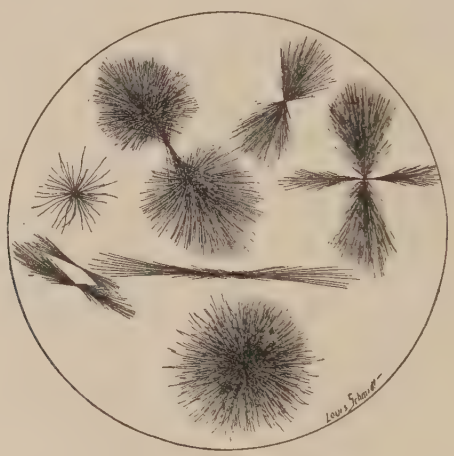
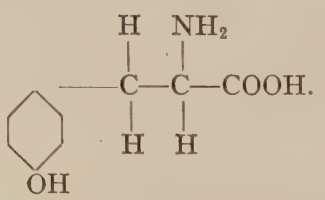


FIG. 46.—TYROSINE.

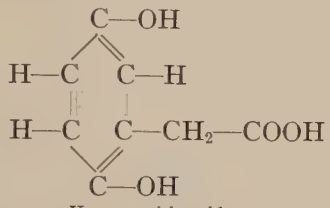
l-**Tryosine,** $C_9H_{11}NO_3$, the least soluble of the amino acids, has the following graphic formula and is *α-amino-β-para-hydroxy-phenyl propionic acid*,



It crystallizes from water in sheath-like groups of fine white needles. It is slightly soluble in water (1 part to 2500 of water at room temperature), quite insoluble in ether, alcohol, acetone and glacial acetic acid but readily soluble in very dilute alkalis and acids. Its melting point is indefinite but is usually given as between 315°–320°. Tyrosine is found in most proteins but is lacking in gelatin. It is thought to be a necessary constituent of protein for a well-blanced diet. Formerly it was believed that tyrosine might be replaced by phenylalanine, as

[1] Shambaugh and Lewis: *Jour. Biol. Chem.*, 67, xxxii, 1926.

tyrosine was considered the first step in the metabolism of phenylalanine, but scientific evidence seems to oppose this view. Tyrosine, in the diabetic organism, is incapable of producing "extra" glucose, but does increase the amount of acetone bodies, and like phenylalanine it also increases the quantity of homogentisic acid excreted in the urine of the patient with alcaptonuria. It would, therefore, seem probable that homogentisic acid is one of the intermediary steps in the metabolism of both phenylalanine and tyrosine.



Homogentisic acid

Tyrosine may be detected in the faintest traces by means of the Millon's reaction. (See p. 162.) For tyrosine crystals see Fig. 46.

Preparation of Tyrosine.—Introduce 200 grams of commercial casein[1] into a 3-liter pyrex flask, add a liter of cold water and shake well. To this mixture add a liter of boiling water, slowly with continuous shaking. Now add 40 c.c. of 20 per cent sodium hydroxide to dissolve the casein and adjust the reaction of the casein solution to pH8 by the addition of normal sodium hydroxide.[2]

Preserve the casein solution and diminish oxidase action by adding 15 c.c. of toluol and 2 gm. of sodium fluoride dissolved in about 10 c.c. of hot water. Shake well, add trypsin in the form of some commercial preparation,[3] and after mixing the contents of the flask thoroughly, stopper the flask and place it in an incubator at 38–40°. Shake the flask thoroughly every day, without removing the stopper, and at the end of the fourth day add an additional quantity of trypsin in one of the forms mentioned. After permitting the digestion to continue for a second period of four days remove the flask from the incubator, allow it to stand at room temperature for at least 24 hours, then filter off the precipitate of tyrosine, undigested casein, etc. Treat the residue with dilute sulphuric acid (5 c.c. concentrated sulphuric acid in 250 c.c. of water) to dissolve the tyrosine, filter through a pleated paper[4] add 10 c.c. concentrated ammonium hydroxide to the filtrate and heat on a boiling water bath. The solution, which should now be acid to litmus, is carefully neutralized by the addition of ammo-

[1] An excellent product may be obtained from The Harris Laboratories, Inc., Tuckahoe. N. Y.

[2] To do this take 10 c.c. of the casein mixture, add 10 drops of cresol red and titrate with 0.2N NaOH (using a microburette or a 1 c.c. pipette graduated in $\frac{1}{100}$ c.c.) until a reddish-purple color is obtained. Multiply this titration volume by 40 and add this quantity of normal sodium hydroxide to the casein solution in the pyrex flask. Shake the mixture at frequent intervals after the hydroxide is added. The reaction should now be acid to phenolphthalein and alkaline to cresol red.

[3] If pure trypsin is not available the enzyme may be added in the form of "pancreatin," "liquor pancreaticus" or the finely divided pancreas of a pig or sheep.

[4] This filtration may be a slow process. In case the first filtrate is turbid pass it back through the paper until clear.

nium hydroxide and allowed to cool. Tyrosine, contaminated with more or less calcium phosphate should crystallize out. Filter off the tyrosine by suction, suspend it in 300 c.c. of water in a flask, heat to boiling, add 5 c.c. of concentrated ammonium hydroxide and boil for 15 minutes. Filter off the insoluble calcium phosphate, neutralize the tyrosine filtrate with 5 per cent H_2SO_4 and allow it to stand. Filter off the tyrosine crystals by suction, wash well with cold water and alcohol in turn, and dry in an oven or incubator. If the tyrosine crystals are not well formed (see Fig. 46, p. 148) they may be recrystallized from hot water. (Solubility 1 : 154.)

Experiments on Tyrosine.—Make the following tests with the tyrosine crystals prepared in the above experiment, or upon some pure tyrosine furnished by the instructor.

1. Microscopical Examination.—Place a minute crystal of tyrosine on a slide, add a drop of water, cover with a cover-glass, and examine microscopically. Now run more water under the cover-glass and warm in a Bunsen flame until the tyrosine has dissolved. Allow the solution to cool slowly, then examine again microscopically, and compare the crystals with those shown in Fig. 46, page 148.

2. Solubility.—Try the solubility of very small amounts of tyrosine in cold and hot water, cold and hot 95 per cent alcohol, dilute NH_4OH, dilute KOH and dilute HCl.

3. Sublimation.—Place a little tyrosine in a dry test-tube, heat gently and notice that the material does not sublime. How does this compare with the result of Experiment 3 (page 134) under Leucine?

4. Hoffman's Reaction.—This is the name given to Millon's reaction when employed to detect tyrosine. Add about 3 c.c. of water and a few drops of Millon's reagent to a little tyrosine in a test-tube. Upon dissolving the tyrosine by heat the solution gradually darkens and may assume a dark red color. What group does this test show to be present in tyrosine?

5. Sulphuric Acid Test (Piria).—Warm a little tyrosine on a watch glass on a boiling water-bath for 20 minutes with 3–5 drops of conc. H_2SO_4. Tyrosine-sulphuric acid is formed in the process. Cool the solution and wash it into a small beaker with water. Now add $CaCO_3$ in substance slowly with stirring, until the reaction of the solution is no longer acid. Filter, concentrate the filtrate, and add it to a few drops (avoid an excess) of very dilute neutral ferric chloride. A purple or violet color, due to the formation of the ferric salt of tyrosine-sulphuric acid, is produced. This is one of the most satisfactory tests for the identification of tyrosine.

6. Formaldehyde-Sulphuric Acid Test (Mörner).—Add about 3 c.c. of Mörner's reagent[1] to a little tyrosine in a test-tube, and gently raise the temperature to the boiling-point. A green color results.

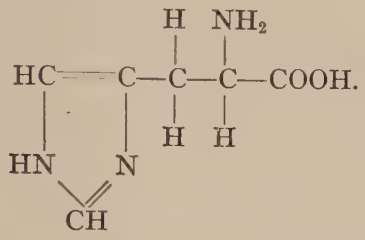
7. Folin and Denis's Test.[2]—To 1–2 c.c. of the solution to be tested add an equal volume of a special reagent (containing 10 per cent sodium tungstate, 2 per cent phosphomolybdic acid and 10 per cent phosphoric acid) and 3–10 c.c. of a saturated solution of sodium carbonate. A blue color indicates tyrosine. It is said to detect 1 part in one million.

[1] Mörner's reagent is prepared by thoroughly mixing 1 volume of formalin, 45 volumes of distilled water, and 55 volumes of concentrated sulphuric acid.

[2] Folin and Denis: *Jour. Biol. Chem.*, 12, 245, 1912.

Abderhalden[1] claims the reagent also reacts with tryptophane, oxytryptophane and *l*-oxyproline.

Histidine, $C_6H_9N_3O_2$, is *α-amino β-imidazole propionic acid* with the following structural formula:



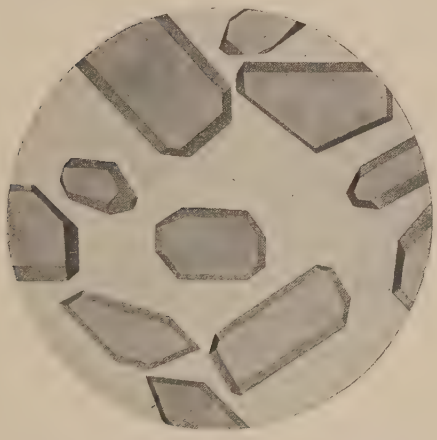


Fig. 47.—Histidine Dihydrochloride.

Histidine is found in small quantities in practically all proteins, varying from 1.5–10 per cent. It is readily soluble in water, giving a strong alkaline reaction. It is only slightly soluble in alcohol but crystallizes from a saturated alcoholic solution in thin platelets which melt at 250°–255°C.

Histidine combines with a variety of substances forming, for example, mono and di-hydrochlorides, also compounds or double salts with platinum chloride or silver nitrate which are particularly valuable for obtaining a pure crystalline compound. It forms, moreover, characteristic compounds with picric acid, phosphotungstic acid, nitrobenzoyl chloride, etc. For crystals of the dihydrochloride see Fig. 47.

Ackroyd and Hopkins[2] reported some highly suggestive experiments in which it seemed that histidine and arginine may function as a source

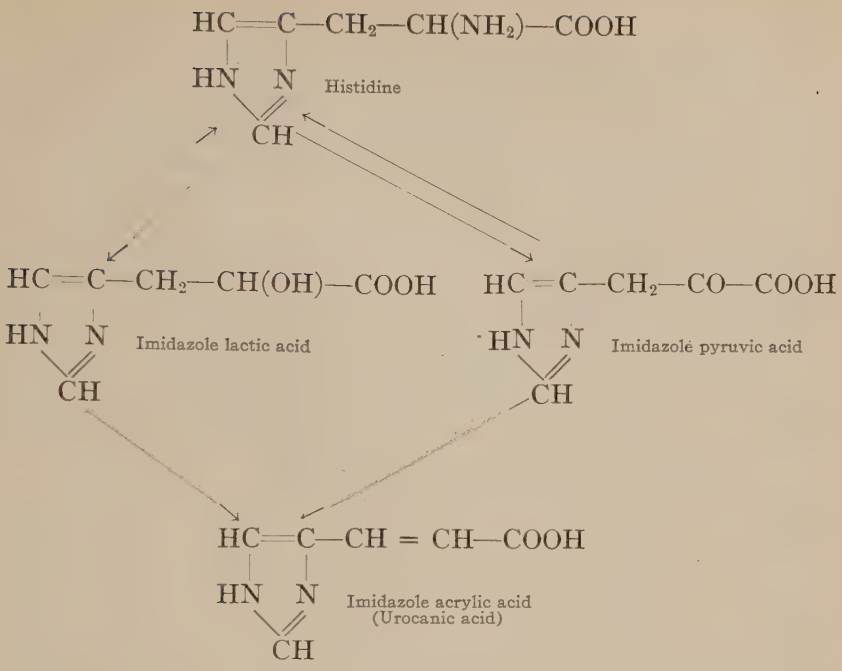
[1] Abderhalden: *Zeit. physiol. Chem.*, 85, 91, 1913.
[2] Ackroyd and Hopkins: *Bioch. Jour.*, 10, 551, 1916.

of purines and a consideration of the structure of these amino acids supports this view. They found that one, but not both, of these amino acids is essential for maintenance and growth of young rats and that in their absence the normal excretion of allantoin was much diminished. Rose and Cook[1] have shown that although histidine is most likely an essential amino acid it cannot be replaced by arginine, thus denying the theory of the interconversion of histidine and arginine. These authors believe that histidine may to some degree add to the daily purine excretion. Novello, Harrow and Sherwin[2] found that imidazole itself is to a large degree oxidized in the human as well as in the animal body and adds considerably to the uric acid output in human urine and to the allantoin output in animal urines. Histidine according to the last named authors apparently contributes its α amino nitrogen to the formation of urea and its nitrogen of the ring to purine derivatives.

The fate of histidine in the body is rather unsettled as it is believed by some to pass through the α-hydroxy acid stage and by others to pass through the α-keto acid stage. Also it has been suggested that it might form imidazole acrylic acid as an intermediary metabolism product since this substance (urocanic acid) has been found in the urine of a few dogs after excessive ingestion of histidine.[3] Harrow and Sherwin[4] find that all three of these substances are catabolized easily in the animal body and therefore might be intermediary products of histidine metabolism. Cox and Rose find[5] that imidazole lactic acid may practically replace histidine in the diet of white rats. This is the first successful demonstration of the substitution of a non-amino compound in the diet for an essential amino acid. Novello, Harrow and Sherwin[2] report similar findings, and also observe that imidazole pyruvic acid has considerable value as a substitute for histidine in the diet, while imidazole acrylic acid has very little. This would indicate that although histidine is an essential amino acid, it may be synthesized from at least two of its physiological degradation products, and that the α, keto or hydroxy stage may be the first step in the metabolism of histidine. These experiments fail to support the idea that α-β unsaturation enters into the normal metabolism of amino acids.

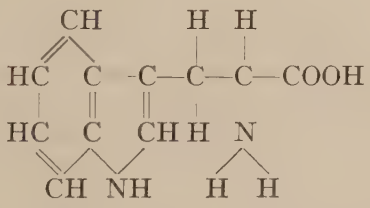
The following scheme may represent the steps in the metabolism of histidine:

[1] Rose and Cook: *Jour. Biol. Chem.*, 64, 325, 1925.
[2] Novello, Harrow and Sherwin: *Jour. Biol. Chem.*, 67, liv, 1926.
[3] Jaffé: *Ber.*, 7, 1669, 1874.
[4] Unpublished data.
[5] Cox and Rose: *Jour. Biol. Chem.*, 67, iii, 1926.



Histidine does not produce glucose and Dakin[1] found no clear evidence that it produced β-hydroxybutyric acid in the diabetic organism.

Tryptophane.—$C_{11}H_{12}NO_2$, has the following graphical formula as shown by Ellinger:[2]



and is therefore *α-amino β-indole propionic acid.* The inactive compound was synthesized by Ellinger and Flamand[3] from indole aldehyde. The natural laevo-rotatory compound is best prepared by the prolonged action of trypsin on casein according to the method of Frankel and Feldsberg.[4] Tryptophane crystallizes in thin shining rhombic and six-sided plates. Fig. 48. It is almost tasteless. It is quite insoluble in cold water, cold and hot absolute alcohol and cold pyridine, but quite soluble in hot water and hot pyridine. The melting point depends

[1] Dakin: *Jour. Biol. Chem.*, 14, 328, 1913.
[2] Ellinger: *Ber.*, 37, 1801, 1904.
[3] Ellinger and Flamand: *Zeit. physiol. Chem.*, 55, 8, 1908.
[4] Frankel and Feldsberg: *Biochem. Zeit.*, 120, 218, 1921.

to a considerable degree upon the rapidity of heating. By slowly heating it is said to change color slightly at 220°, to turn brown at 240° and melt completely at 252°. Tryptophane is yielded by nearly all proteins, but is present in very small amounts in gelatin[1] and in zein, the protein of maize. Tryptophane is present in maximum amount in lactalbumin.

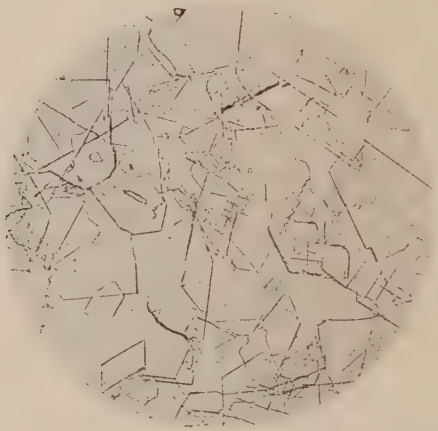


FIG. 48.—TRYPTOPHANE. (From Keenan, *Jour. Biol. Chem.*, 62, 163, 1924.)

Color Reactions for Tryptophane.—(a) Hopkins-Cole Test.—If a substance containing this amino acid is placed in a test tube, and a solution containing a small amount of glyoxylic acid is added, then sulphuric acid stratified on the bottom of the tube, a reddish violet ring will appear at the juncture of the two liquids. This is also known as the glyoxylic acid test. **(See p. 163.)**

(b) Aldehyde Reactions.—Tryptophane will also give color reactions in the presence of aromatic aldehydes. With p-dimethylaminobenzaldehyde in sulphuric acid it gives a red violet color. These color reactions are apparently due to the presence of the indole ring in tryptophane. The indole ring, being a combination of the benzene and pyrrole rings, probably owes its chromogenic properties to the latter ring. For other tryptophane reactions see p. 260.

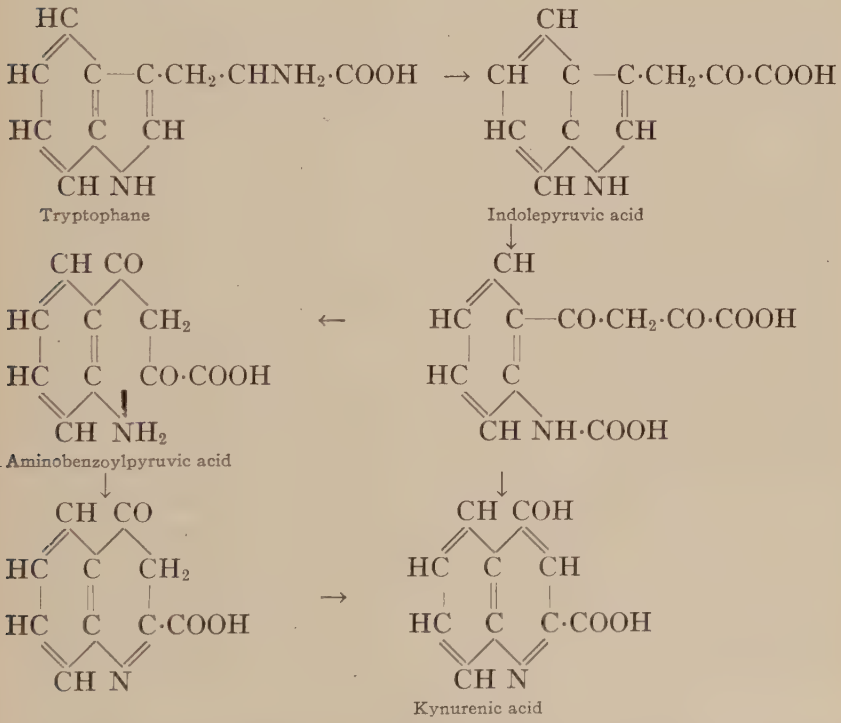
Colorimetric Determination of Tryptophane. Method of Kraus.[1]—To 0.2–1.0 mg. tryptophane in 2 c.c. of water add 0.4 c.c. of 0.5 per cent vanillin in 50 per cent acetic acid, and 15 c.c. of conc. HCl. Mix, let stand for 24 hours, dilute to 50 c.c. and compare with a standard. Indole and skatole interfere but can be extracted with toluene.

From a nutritional standpoint, tryptophane is one of the most important amino acids. Maintenance of body weight and function may be continued for a long time with cessation of growth when zein and tryptophane are fed, but only when lysine is added is growth re-

[1] Kraus: *Jour. Biol. Chem.*, 63, 157, 1925.

sumed.[1] If zein is fed alone without either tryptophane or lysine, there is not only cessation of growth but a failure to maintain a condition of health and normal body function. It was thought that tryptophane might be a precursor of thyroxin, but this has been disproved in the recent work of Hsi-Chung Chang.[2]

The indole ring is resistant to oxidation in the body, but like the benzene ring is easily oxidized when combined with alanine. The intermediary metabolism of tryptophane is still in doubt, although it seems probable that kynurenic acid is one of the intermediary products. Heft and Sherwin[3] have found that neither indolepyruvic acid, indolelactic acid nor kynurenic acid can replace tryptophane in the diet. Ellinger[4] suggested the following as a possible pathway in the catabolism of tryptophane:

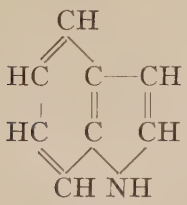


Tryptophane unlike most of the protein constituents forms neither glucose nor β-hydroxybutyric acid in the diabetic organism.

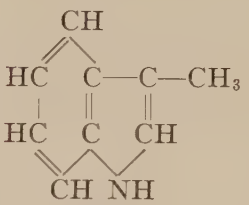
Intestinal bacteria act upon the propionic acid chain of the compound, converting tryptophane into indole and skatole, which are

[1] Mendel: *Jour. Am. Med. Assn.*, 64, 1539, 1915.
[2] Hsi-Chung Chang: *Am. Jour. Physiol.*, 73, 275, 1925.
[3] Unpublished data.
[4] Ellinger and Matsuoka: *Zeit. physiol. Chem.*, 91, 45, 1914.

conjugated with sulphuric acid or glycuronic acid and excreted in the urine.



Indole



Skatole

CHAPTER VI

PROTEINS: THEIR CLASSIFICATION AND PROPERTIES

From what has already been said in Chapter V regarding the protein substances it will be recognized that the grouping of the diverse forms of this class of substances in a logical manner is not an easy task. The fats and carbohydrates may be classified upon the fundamental principles of their stereo-chemical relationships, whereas such a system of classification in the case of the proteins is absolutely impossible since, as we have already stated, the molecular structure of these complex substances is unknown. Because of the diversity of standpoints from which the proteins may be viewed, relative to their grouping in the form of a logically classified series, it is obvious that there is an opportunity for the presentation of classifications of a widely divergent character. The fact that there were until recent years at least a dozen different classifications which were recognized by various groups of English-speaking investigators emphasizes the difficulties in the way of the individual or individuals who would offer a classification which should merit universal adoption. Realizing the great handicap and disadvantage which the great diversity of the protein classifications was forcing upon the workers in this field, the Chemical and Physiological Societies of England drafted a classification which appealed to these groups of scientists as fulfilling all requirements and presented it for the consideration of the American Physiological Society and the American Society of Biological Chemists. The outcome of this has been that there are now only *two* protein classifications which are recognized by English-speaking scientists, one the British Classification, the other the American Classification. These classifications are very similar and doubtless will ultimately be merged into a single classification. In our consideration of the proteins we shall conform in all details to the American Classification. In this connection we will say, however, that we feel that the English Societies have strong grounds for preferring the use of the term *scleroproteins* for albuminoids and *chromoproteins* for hemoglobins. The two classifications are as follows:

CLASSIFICATION OF PROTEINS ADOPTED BY THE AMERICAN PHYSIOLOGICAL SOCIETY AND THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

I. SIMPLE PROTEINS

Protein substances which yield *only α-amino acids* or their derivatives on hydrolysis.

(*a*) **Albumins.**—Soluble in pure water and coagulable by heat, *e.g.*, *ovalbumin* from *egg white*, *serum albumin* from *blood serum*, *lactalbumin* from *milk*, *vegetable albumins*.

(*b*) **Globulins.**—Insoluble in pure water but soluble in neutral solutions of salts of strong bases with strong acids,[1] *e.g.*, *serum globulin*, *ovoglobulin* from *egg yolk*, *edestin* from *hemp seed*, *amandin* from *almond* and *peach kernel*, and other *vegetable globulins*.

(*c*) **Glutelins.**—Simple proteins insoluble in *all neutral solvents*, but readily soluble in very dilute acids and alkalis,[2] *e.g.*, *glutenin* from *wheat*.

(*d*) **Alcohol-soluble Proteins (Prolamins).**[3]—Simple proteins soluble in 70–80 per cent alcohol, insoluble in water, absolute alcohol, and other *neutral solvents*,[4] *e.g.*, *zein* from *corn*, *gliadin* from *wheat* and *rye*, *hordein* from *barley*, and *bynin* from *malt*. *Rice*,[5] *kafir*[6] and *sorghum*[7] have also been shown to contain alcohol-soluble proteins.

(*e*) **Albuminoids.**—Simple proteins possessing a similar structure to those already mentioned, but characterized by a pronounced insolubility in all neutral solvents,[8] *e.g.*, *elastin* from *ligament*, *collagen* from *tendon*, *keratin* from *horn* and *hoof*.

(*f*) **Histones.**—Soluble in water and insoluble in very dilute ammonia, and, in the absence of ammonium salts, insoluble even in excess of ammonia; yield precipitates with solutions of other proteins and a coagulum on heating which is easily soluble in very dilute acids. On hydrolysis they yield a large number of amino acids among which the basic ones predominate. In short, histones are basic proteins which stand

[1] The precipitation limits with ammonium sulphate should not be made a basis for distinguishing the albumins from the globulins.

[2] Such substances occur in abundance in the seeds of cereals and doubtless represent a well-defined natural group of simple proteins.

[3] The name *prolamins* has been suggested for these alcohol-soluble proteins by Dr. Thomas B. Osborne (*Science*, 28, 417, 1908). It is a very fitting term inasmuch as upon hydrolysis they yield particularly large amounts of *proline and ammonia*.

[4] The subclasses defined (*a*, *b*, *c*, *d*,) are exemplified by proteins obtained from both plants and animals. The use of appropriate prefixes will suffice to indicate the origin of the compounds, *e.g.*, *ovo*globulin, *lact*albumin, etc.

[5] Hoffman: *Jour. Biol. Chem.*, 66, 501, 1925.

[6] Johns and Brewster: *Jour. Biol. Chem.*, 28, 59, 1916.

[7] Hoffman and Gortner: Colloid Symposium Monograph, 1924, ii, Chap. 20.

[8] These form the principal organic constituents of the skeletal structure of animals and also their external covering and its appendages. This definition does not provide for gelatin which is, however, an artificial derivative of collagen.

between protamines and true proteins, *e.g.*, *globin* from *hemoglobin*, *scombrone* from *mackerel sperm*, *thymus histone*.

(*g*) **Protamines.**—Simpler polypeptides than the proteins included in the preceding groups. They are soluble in water, uncoagulable by heat, have the property of precipitating aqueous solutions of other proteins, possess strong basic properties and form stable salts with strong mineral acids. They yield comparatively few amino acids, among which the basic ones predominate. They are the *simplest natural proteins*, *e.g.*, *salmine* from *salmon sperm*, *sturine* from *sturgeon sperm*, *clupeine* from *herring sperm*, *scombrine* from *mackerel sperm*.

II. CONJUGATED PROTEINS

Substances which contain the protein molecule united to some other molecule or molecules otherwise than as a salt.

(*a*) **Nucleoproteins.**—Compounds of one or more protein molecules with nucleic acid, *e.g.*, *cytoglobulin* from *cytoplasm*, *nucleohistone* from *nucleus*.

(*b*) **Glycoproteins.**—Compounds of the protein molecule with a substance or substances containing a carbohydrate group other than a nucleic acid, *e.g.*, *mucins* and *mucoids* (*osseomucoid* from *bone*, *tendomucoid* from *tendon*, *ichthulin* from *carp eggs*, *helicoprotein* from *snail*).

(*c*) **Phosphoproteins.**—Compounds of the protein molecule with some, as yet undefined, phosphorus-containing substances other than a nucleic acid or lecithin,[1] *e.g.*, *casein* from *milk*, *ovovitellin* from *egg yolk*.

(*d*) **Hemoglobins.**—Compounds of the protein molecule with hematin, or some similar substance, *e.g.*, *hemoglobin* from *red blood cells*, *hemocyanin* from *blood of invertebrates*.

(*e*) **Lecithoproteins.**—Compounds of the protein molecule with *lecithins*.

III. DERIVED PROTEINS

1. Primary Protein Derivatives

Derivatives of the protein molecule apparently formed through hydrolytic changes which involve only slight alteration of the protein molecule.

(*a*) **Proteans.**—Insoluble products which apparently result from the incipient action of water, very dilute acids or enzymes, *e.g.*, *myosan* from *myosin*, *edestan* from *edestin*.

[1] The accumulated chemical evidence distinctly points to the propriety of classifying the phosphoproteins as conjugated compounds, *i.e.*, they are possibly esters of some phosphoric acid or acids and protein.

(*b*) **Metaproteins.**—Products of the further action of acids and alkalis whereby the molecule is so far altered as to form products soluble in very weak acids and alkalis but insoluble in neutral fluids, *e.g.*, *acid metaprotein* (*acid albuminate*), *alkali metaprotein* (*alkali albuminate*).

(*c*) **Coagulated Proteins.**—Insoluble products which result from (1) the action of heat on their solutions, or (2) the action of alcohol on the protein.

2. Secondary Protein Derivatives[1]

Products of the further hydrolytic cleavage of the protein molecule.

(*a*) **Proteoses.**—Soluble in water, non-coagulable by heat, and precipitated by saturating their solutions with ammonium—or zinc sulphate,[2] *e.g.*, *protoproteose*, *deuteroproteose.*

(*b*) **Peptones.**—Soluble in water, non-coagulable by heat, but *not precipitated* by saturating their solutions with ammonium sulphate,[3] *e.g.*, *antipeptone*, *amphopeptone.*

(*c*) **Peptides.**—Definitely characterized combinations of two or more amino acids, the *carboxyl* group of one being united with the *amino* group of the other with the elimination of a molecule of water,[4] *e.g.*, *dipeptides*, *tripeptides*, *tetrapeptides*, *pentapeptides.*

CLASSIFICATION OF PROTEINS ADOPTED BY THE CHEMICAL AND PHYSIOLOGICAL SOCIETIES OF ENGLAND

I. Simple Proteins

1. Protamines, *e.g.*, *salmine*, *clupeine.*
2. Histones, *e.g.*, *globin*, *scombrone.*
3. Albumins, *e.g.*, *ovalbumin*, *serum albumin*, *vegetable albumins.*
4. Globulins, *e.g.*, *serum globulin*, *ovoglobulin*, *vegetable globulins.*
5. Glutelins, *e.g.*, *glutenin.*
6. Alcohol-soluble proteins, *e.g.*, *zein*, *gliadin.*
7. Scleroproteins, *e.g.*, *elastin*, *keratin.*
8. Phosphoproteins, *e.g.*, *casein*, *vitellin.*

[1] The term secondary protein derivatives is used because the formation of the primary derivatives usually precedes the formation of the secondary derivatives.

[2] As thus defined, this term does not strictly cover all the protein derivatives commonly called proteoses, *e.g.*, heteroproteose and dysproteose.

[3] In this group the kyrines may be included. For the present it is believed that it will be helpful to retain this term as defined, reserving the expression *peptide* for the simpler compounds of definite structure, such as dipeptides, etc.

[4] The peptones are undoubtedly peptides or mixtures of peptides, the latter term being at present used to designate those of definite structure.

II. Conjugated Proteins

1. Glucoproteins, *e.g.*, *mucins*, *mucoids*.
2. Nucleoproteins, *e.g.*, *nucleohistone*, *cytoglobulin*.
3. Chromoproteins, *e.g.*, *hemoglobin*, *hemocyanin*.

III. Products of Protein Hydrolysis

1. Infraproteins, *e.g.*, *acid infraprotein* (*acid albuminate*), *alkali infraprotein* (*alkali albuminate*).
2. Proteoses, *e.g.*, *protoproteose*, *heteroproteose*, *deuteroproteose*.
3. Peptones, *e.g.*, *amphopeptone*, *antipeptone*.
4. Polypeptides, *e.g.*, *dipeptides*, *tripeptides*, *tetrapeptides*.

CONSIDERATIONS OF THE VARIOUS CLASSES OF PROTEINS

SIMPLE PROTEINS

The simple proteins are true protein substances which, upon hydrolysis, yield *only* α-amino acids or their derivatives. "Although no means are at present available whereby the chemical individuality of any protein can be established, a number of simple proteins have been isolated from animal and vegetable tissues which have been so well characterized by constancy of ultimate composition and uniformity of physical properties that they may be treated as chemical individuals until further knowledge makes it possible to characterize them more definitely." Under simple proteins we may class albumins, globulins, glutelins, prolamins, albuminoids, histones and protamines. Several proteins have been obtained in crystalline form, notably, egg albumin, serum albumin, Bence-Jones protein, edestin, excelsin and other vegetable globulins. Recent additions to crystalline proteins include one with tuberculin activity,[1] a peculiar urinary protein,[2] and a globulin considered to be identical with the enzyme urease (see pages 46 and 64).

The researches of the late Jacques Loeb[3] in which applications of hydrogen ion determinations and the Donnan equilibrium (see p. 4) were made in studies of protein solutions, have resulted in a widespread tendency to regard proteins as definite chemical bodies of amphoteric nature, behaving in accordance with the classical laws of chemistry. Thus the cloud of uncorrelated data hitherto ascribed to the colloidal nature of proteins appears to be lifting.

[1] Seibert: *Science*, 63, 619, 1926.
[2] Hektoen, Kretschmer and Welker: *Jour. Am. Med. Assn.*, 83, 1154, 1924.
[3] Loeb: Proteins and the Theory of Colloidal Behavior, New York, 1925, 2nd Ed.

ALBUMINS

Albumins constitute the first class of simple proteins and may be defined as simple proteins which are coagulable by heat and soluble in pure (salt-free) water. They are also soluble in salt solutions and those of animal origin are not precipitated upon saturating their *neutral* solutions at 30°C. with sodium chloride or magnesium sulphate, but if a saturated solution of this character be acidified with acetic acid the albumin precipitates. All albumins of *animal* origin may be precipitated by saturating their solutions with ammonium sulphate.[1] They may be thrown out of solution by the addition of a sufficient quantity of a mineral acid, whereas a weak acidity produces a slight precipitate which dissolves upon agitating the solution. Metallic salts also possess the property of precipitating albumins, some of the precipitates being soluble in excess of the reagent, whereas others are insoluble in such an excess. Of those proteins which occur native the albumins contain the highest percentage of sulphur, ranging from 1.6 to 2.5 per cent. Some albumins have been obtained in crystalline form, notably egg albumin, serum albumin, and lactalbumin, but the fact that they may be obtained in crystalline form does not necessarily prove them to be chemical individuals.

GENERAL COLOR REACTIONS OF PROTEINS

These color reactions are due to a reaction between some one or more of the constituent radicals or groups of the complex protein molecule and the chemical reagent or reagents used in any given test. Not all proteins contain the same groups and for this reason the various color tests will yield reactions varying in intensity of color according to the nature of the groups contained in the particular protein under examination. Various substances not proteins respond to certain of these color reactions, and it is therefore essential to submit the material under examination to several tests before concluding definitely regarding its nature.

TECHNIC OF THE COLOR REACTIONS

1. Millon's Reaction.—To 5 c.c. of a dilute solution of egg albumin[2] in a test-tube add a few drops of Millon's reagent. A white precipitate forms which turns red when heated.

[1] In this connection, Osborne's observation that there are certain *vegetable* albumins which are precipitated by saturating their solutions with sodium chloride or magnesium sulphate or by half-saturating with ammonium sulphate, is of interest.

[2] This egg albumin solution may be prepared by beating egg-white with 6–10 volumes of water. The precipitate of ovoglobulin is filtered off and the filtrate used in the tests.

This test is a particularly satisfactory one for use on *solid* proteins, in which case the reagent, diluted with water 1:5, is added directly to the solid substance and heat applied, which causes the substance to assume a red color. Such proteins as are not precipitated by mineral acids, for example certain of the proteoses and peptones, yield a *red solution* instead of a red precipitate.

The reaction is due to the presence of the *hydroxy-phenyl group*, $-C_6H_4OH$, in the protein molecule and certain non-proteins such as tyrosine, phenol (carbolic acid) and thymol also respond to the reaction. Inasmuch as the tyrosine grouping is the only hydroxyphenyl grouping which has definitely been proven to be present in the protein molecule it is evident that protein substances respond to Millon's reaction because of the presence of this tyrosine complex. The test is not a very satisfactory one for use in solutions containing inorganic salts in large amount, since the mercury of the Millon's reagent[1] is thus precipitated and the reagent rendered inert. This reagent is therefore never used for the detection of protein material in the urine. If the solution under examination is strongly alkaline it should be neutralized inasmuch as the alkali will precipitate yellow or black oxides of mercury.

2. Xanthoproteic Reaction.—To 2–3 c.c. of egg albumin solution in a test-tube add concentrated nitric acid. A white precipitate forms, which upon heating turns yellow and finally dissolves, imparting to the solution a yellow color. Cool the solution and carefully add ammonium hydroxide, potassium hydroxide, or sodium hydroxide in excess. Note that the yellow color deepens into an orange.

This reaction is due to the presence in the protein molecule of the *phenyl group*—C_6H_5, with which the nitric acid forms certain nitro modifications. The particular complexes of the protein molecule which are of especial importance in this connection are those of tyrosine, phenylalanine, and tryptophane. The test is not a satisfactory one for use in urinary examination because of the color of the end-reaction.

3. Glyoxylic Acid Reaction (Hopkins-Cole).[2]—Place 1–2 c.c. of egg albumin solution and 3 c.c. of glyoxylic acid, CHO.COOH + H_2O or $CH(OH)_2COOH$, solution (Hopkins-Cole reagent[3]) in a test-tube and mix thoroughly. In a second tube place 5 c.c. of concentrated sulphuric acid. Incline the tube containing sulphuric acid and by means of a pipette allow the albumin-glyoxylic acid solution to flow carefully down the side. When stratified in this manner a reddish-violet color forms at the zone of contact of the two fluids.

[1] Millon's reagent consists of mercury dissolved in nitric acid containing some nitrous acid. It is prepared by digesting one part (by weight) of mercury with two parts (by weight) of HNO_3 (sp. gr 1.42) and diluting the resulting solution with two volumes of water.

[2] Hopkins and Cole: *Journal of Physiology*, 27, 418, 1902.

[3] Hopkins-Cole reagent is prepared as follows: To one liter of a saturated solution of oxalic acid add 60 grams of sodium amalgam and allow the mixture to stand until the evolution of gas ceases. Filter and dilute with 2–3 volumes of water.

In performing the test on a solid substance employ modification described on page 172.

This color is due to the presence of the *tryptophane group*. Gelatin does not respond to this test. For formula of tryptophane see page 66. Benedict[1] has suggested a new reagent for use in carrying out the Hopkins-Colere action.[2] Nitrates ($NaNO_3$ and KNO_3) chlorates, nitrites, or excess of chlorides, entirely prevent the reaction.[3] The sulphuric acid used must be pure.

4. Biuret Test.—To 2–3 c.c. of egg albumin solution in a test-tube add an equal volume of concentrated potassium hydroxide solution, mix thoroughly, and add slowly a very dilute (2–5 drops in a test-tube of water) copper sulphate solution until a purplish-violet or pinkish-violet color is produced. The depth of the color depends upon the nature of the protein; proteoses, and peptones giving a decided pink, while the color produced with gelatin is not far removed from a blue.

This reaction is given by those substances which contain *two amino groups* in their molecule, these groups either being joined directly together or through a single atom of nitrogen or carbon. The amino groups mentioned must either be two $CONH_2$ groups or one $CONH_2$ group and one $CSNH_2$, $C[NH]NH_2$ or CH_2NH_2 group. It follows from this fact that substances which are non-protein in character but which contain the necessary groups will respond to the biuret test. As examples of such substances may be cited *oxamide*.

and *biuret*,

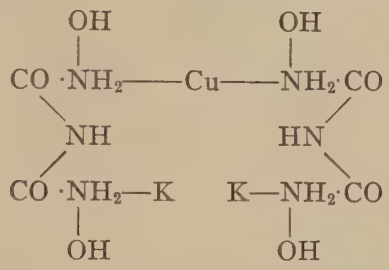
[1] Benedict: *Journal of Biological Chemistry*, 6, 51, 1909.

[2] Benedict's modified Hopkins-Cole reagent is prepared as follows: Ten grams of powdered magnesium are placed in a large Erlenmeyer flask and shaken up with enough distilled water to liberally cover the magnesium. Two hundred and fifty c.c. of a cold, saturated solution of oxalic acid is now added slowly. The reaction proceeds very rapidly and with the liberation of much heat, so that the flask should be cooled under running water during the addition of the acid. The contents of the flask are shaken after the addition of the last portion of the acid and then poured upon a filter, to remove the insoluble magnesium oxalate. A little wash water is poured through the filter, the filtrate acidified with acetic acid to prevent the partial precipitation of the magnesium on long standing, and made up to a liter with distilled water. This solution contains only the magnesium salt of glyoxylic acid.

[3] Mathewson: Dissertation (Columbia Univ.), Eschenbach Publishing Co., Easton, Pa., 1912. Cole: *Practical Physiological Chemistry*, 7th Ed., 1926, p. 87.

The test derives its name from the fact that this latter substance which is formed on heating urea to 180°C. will respond to the test. Protein material responds positively since there are two $CONH_2$ groups in the protein molecule.

According to Schiff the end-reaction of the biuret test is dependent upon the formation of a copper-potassium-biuret compound (cupri-potassium biuret or biuret potassium cupric hydroxide). This substance was obtained by Schiff in the form of long red needles. It has the following formula:



If much magnesium sulphate is present a precipitate of magnesium hydroxide forms which interferes with the test. If considerable ammonium sulphate is present a large excess of alkali must be used.

Testing Colored Solutions by Biuret Test.—If the color of the solution is such as to interfere with the end-reaction of the biuret test, proceed as follows: Make the solution strongly alkaline with potassium hydroxide and add a solution of copper sulphate. Shake up the mixture with alcohol and if protein is present the alcohol will assume the typical biuret coloration. This procedure is not applicable in case the pigment of the original solution is soluble in alcohol. Excess of the copper salt need not be avoided in this test.

Gies's Biuret Reagent.[1]—Gies has devised a reagent for use in the biuret test. This reagent consists of 10 per cent KOH solution, to which 25 c.c. of 3 per cent $CuSO_4$ solution per liter has been added. This imparts a slight though distinct blue color to the clear liquid. This reagent is of material assistance in performing the biuret test.

Biuret Paper of Kantor and Gies.—According to Kantor and Gies[2] when filter paper is immersed in the above reagent and subsequently dried it forms a very satisfactory "biuret paper" which may be used in a manner analogous to indicator papers. Moist papers may be used in the examination of powders which are neutral or alkaline in reaction. In preparing the "biuret paper," if the filter paper is left for a sufficient length of time in the reagent all traces of the copper sulphate will be removed from the solution.

[1] Gies: Proceedings of Society of Biological Chemists, *Journal of Biological Chemistry*, 7, 60, 1910.

[2] Kantor and Gies: *Proc. Soc. Biol. Chem.*, p. 11, 1910.

5. Ring Biuret Test (Posner).—This test is particularly satisfactory for use on *dilute* protein solutions, and is carried out as follows. To some dilute egg albumin in a test-tube add one-half its volume of potassium hydroxide solution. Now hold the tube in an inclined position and allow some very dilute copper sulphate solution, made as suggested on page 164, to flow down the side, being especially careful to prevent the fluids from mixing. At the juncture of the two solutions the typical end-reaction of the biuret test should appear as a colored zone (see Biuret Test, page 164).

6. The Triketohydrindene Hydrate (Ninhydrin) Reaction.—To 5 c.c. of dilute protein solution add one-half c.c. of a 0.1 per cent solution of triketohydrindene hydrate, heat to boiling for one to two minutes and allow to cool. A blue color develops if the test is positive.

This test gives positive results with proteins, peptones, peptides, and amino acids which possess a free carboxyl and α-amino group. In a concentration of 1 per cent the ammonium salts of weak acids react positively, as do also the ammonium salts of strong acids in very high concentration. Certain amines also give the reaction.[1]

PRECIPITATION REACTIONS AND OTHER PROTEIN TESTS

There are three forms in which proteins may be precipitated, *i.e.*, *unaltered*, as an *albuminate*, and as an *insoluble salt*. An instance of the precipitation in a *native* or *unaltered* condition is seen in the so-called *salting-out* experiments. Various salts, notably $(NH_4)_2SO_4$, $ZnSO_4$, $MgSO_4$, Na_2SO_4 and NaCl, possess the power, when added in *solid form* to certain definite protein solutions, of rendering the menstruum incapable of holding the protein in solution, thereby causing the protein to be precipitated or *salted-out*, to use the common term. Mineral acids and alcohol also precipitate proteins unaltered. In the case of concentrated acids the protein is dissolved in the presence of an excess of acid with the formation of a protein salt. Proteins are precipitated as *albuminates* when treated with certain metallic salts, and precipitated as *insoluble salts* when weak organic acids such as certain of the alkaloidal reagents are added to their solutions.

If certain acids (picric, phosphotungstic, phosphomolybdic, tannic, or chromic) be added to a *neutral* albumin solution, a precipitate of an *insoluble protein salt* occurs. If, however, the salts of these acids be added no precipitate occurs. The addition of a small amount of acid, as acetic acid, to such a solution will cause a precipitate to form.[2]

The effect of the addition of the salts of the heavy metals is in the first instance to cause a precipitation of the protein. In many cases, however, the addition of an excess of such salts causes the solution of

[1] Harding and Warnerford: *Jour. Biol. Chem.*, 25, 319, 1916.
Harding and MacLean: *Jour. Biol. Chem.*, 25, 337, 1916.
[2] Mathews: *Amer. Jour. of Physiology*, 1, 445, 1898.

the precipitate, while a further excess may cause a reprecipitation. The precipitate which is first formed in a protein solution by the addition of the salts of the heavy metals may be redissolved not only by an excess of such salts but by an excess of protein as well.[1]

Colloidal iron, kaolin and alumina cream are frequently used for removing proteins from solution. The process is one of adsorption and has been adapted to certain quantitative methods.

It is generally stated that globulins are precipitated from their solutions upon *half* saturation with ammonium sulphate and that albumins are precipitated upon *complete* saturation by this salt. Comparatively few exceptions were found to this rule until proteins of *vegetable* origin came to be more extensively studied. These studies, furthered especially by Osborne and associates, have demonstrated very clearly that the characterization of a globulin as a protein which is precipitated by *half* saturation with ammonium sulphate, can no longer hold. Certain vegetable globulins have been isolated which are not precipitated by this salt until a concentration is reached *greater than that secured by half-saturation.* As an example of an albumin which does not conform to the definition of an albumin as regards its precipitation by ammonium sulphate may be mentioned the *leucosin* of the wheat germ, which is precipitated from its solution upon *half*-saturation with ammonium sulphate. The limits of precipitation by ammonium sulphate, therefore, do not furnish a sufficiently accurate basis for the differentiation of globulins from albumins. It has further been determined that a given protein which is precipitable by ammonium sulphate cannot be "salted-out" by the same concentration of the salt under all conditions.

Experiments

1. Influence of Concentrated Mineral Acids, Alkalis and Organic Acids.—Prepare five test-tubes each containing 5 c.c. of concentrated egg albumin solution. To the first add concentrated H_2SO_4, drop by drop, until an excess of the acid has been added. Note any changes which may occur in the solution. Allow the tube to stand for 24 hours and at the end of that period observe any alteration which may have taken place. Heat the tube and note any further change which may occur. Repeat the experiment in the four remaining tubes with concentrated hydrochloric acid, concentrated nitric acid, concentrated potassium hydroxide and acetic acid. How do strong mineral acids, strong alkalis, and strong organic acids differ in their action toward protein solutions?

2. Precipitation by Metallic Salts.—Prepare six tubes each containing 2-3 c.c. of dilute egg albumin solution. To the first add mercuric chloride, drop by drop slowly, until an excess of the reagent has been added, noting any changes which may occur. If not added very gradually the formation of the precipitate

[1] Pauli: *Hofmeister's Beitrage*, 6, 233, 1904–05; Robertson: *Ergebnisse der Physiologie*, 10, 290, 1910.

may not be noted, due to its solubility in excess of the reagent. Repeat the experiment with lead acetate, silver nitrate, copper sulphate, ferric chloride, and barium chloride, using very dilute solutions.

Egg albumin is used as an antidote for lead or mercury poisoning. Why? Is it an equally good antidote for the other metallic salts tested?

3. Precipitation by Alkaloidal Reagents.—Prepare six tubes each containing 2–3 c.c. of dilute egg albumin solution. To the first add picric acid drop by drop until an excess of the reagent has been added, noting any changes which may occur. Repeat the experiment with trichloracetic acid, tannic acid, phosphotungstic acid, phosphomolybdic acid, and potassio-mercuric iodide. Are these precipitates soluble in excess of the reagent? Acidify with hydrochloric acid before testing with the last three reagents.

4. Nitric Acid Test (Heller).—Place 5 c.c. of concentrated nitric acid in a test-tube, incline the tube, and by means of a pipette allow the dilute albumin solution to flow slowly down the side. The liquids should stratify with the formation of a white zone of precipitated albumin at the point of juncture. This is a very delicate test and is further discussed in Chapter XXVIII.

5. Nitric Acid-$MgSO_4$ Test (Roberts).—Place 5 c.c. of Roberts' reagent[1] in a test-tube, incline the tube, and by means of a pipette allow the albumin solution to flow slowly down the side. The liquids should stratify with the formation of a white zone of precipitated albumin at the point of juncture. This test is a modification of Heller's ring test and is rather more satisfactory. The albumoscope may also be used in making this test (see Fig. 196).

6. Tanret's Test.—To 5 c.c. of albumin solution in a test-tube add Tanret's reagent,[2] drop by drop, until a turbidity or precipitate forms. This is an exceedingly delicate test. Sometimes the albumin solution is stratified upon the reagent as in Heller's or Roberts' ring tests.

7. Sodium Chloride and Acetic Acid Test.—Mix 2 volumes of albumin solution and 1 volume of a saturated solution of sodium chloride in a test-tube, acidify with acetic acid, and heat to boiling. The production of a cloudiness or the formation of a precipitate indicates the presence of albumin.

8. Acetic Acid and Potassium Ferrocyanide Test.—To 5 c.c. of dilute egg albumin solution in a test-tube add 5–10 drops of acetic acid. Mix well and add potassium ferrocyanide, drop by drop, until a precipitate forms. This test is very delicate.

Schmiedl claims that a precipitate of $K_2ZnFe(CN)_6$ or $Zn_2Fe(CN)_6$, is formed when solutions containing zinc are subjected to this test, and that this precipitate resembles the precipitate secured with protein solutions. In the case of human urine a reaction was obtained when 0.000022 gram of zinc per cubic centimeter was present. Schmiedl further found that the urine collected from rabbits housed in zinc-lined

[1] Roberts' reagent is composed of 1 volume of concentrated HNO_3 and 5 volumes of a saturated solution of $MgSO_4$.

[2] Tanret's reagent is prepared as follows: Dissolve 1.35 grams of mercuric chloride in 25 c.c. of water, add to this solution 3.32 grams of potassium iodide dissolved in 25 c.c. of water, then make the total solution up to 60 c.c. with water and add 20 c.c. of glacial acetic acid to the combined solutions.

cages possessed a zinc content which was sufficient to yield a ready response to the test. Zinc is the only interfering substance so far reported.

9. Salting-out Experiments.—(a) To 25 c.c. of egg albumin solution in a small beaker add solid ammonium sulphate to the point of saturation, keeping the temperature of the solution below 40°C. Filter, test the precipitate by Millon's reaction and the filtrate by the biuret test. What are your conclusions? (b) Repeat the above experiment, making the saturation with solid sodium chloride. How does this result differ from the result of the saturation with ammonium sulphate? Add 2–3 drops of acetic acid. What occurs?

All proteins *except peptones* are precipitated by saturating their solutions with ammonium sulphate. Most *globulins* are precipitated by saturating their solutions with sodium chloride (see Globulins, page 173); serum globulin, however, is not thus precipitated. If the saturated solution is subsequently acidified, all proteins *except peptones* are precipitated.

Soaps may be salted-out in a similar manner (see page 252).

10. Coagulation or Boiling Test.—Heat 25 c.c. of dilute egg albumin solution to the boiling-point in a small evaporating dish. The albumin coagulates. Complete coagulation may be obtained by acidifying the solution with 3–5 drops of acetic acid[1] at the boiling-point. Test the coagulum by Millon's reaction.

The acid is added to neutralize any possible alkalinity of the solution, to dissolve any substances which are not albumin and to facilitate coagulation (see further discussion on page 182 and in Chapter XXVIII).

11. Coagulation Temperature.—Prepare four test-tubes each containing 5 c.c. of neutral egg albumin solution. To the first add 1 drop of 0.2 per cent hydrochloric acid, to the second add 1 drop of 0.5 per cent sodium carbonate solution, to the third add 1 drop of 10 per cent sodium chloride solution and leave the fourth neutral in reaction. Partly fill a breaker of medium size with water and place it within a second larger beaker which also contains water, the two vessels being separated by pieces of cork. Fasten the four test-tubes compactly together by means of a rubber band, lower them into the water of the inner beaker and suspend them, by means of a clamp attached to one of the tubes, in such a manner that the albumin solutions shall be midway between the upper and lower surfaces of the water. In one of the tubes place a thermometer with its bulb entirely beneath the surface of the albumin solution (Fig. 49). Gently heat the water in the beakers, noting carefully any changes which may occur in the albumin solutions and record the exact temperature at which these changes occur. The first appearance of an opacity in an albumin solution indicates the commencement of coagulation and the temperature at which this occurs should be recorded as the coagulation temperature for that particular albumin solution.

What is the order in which the four solutions coagulate?

[1] Nitric acid is often used in place of acetic acid in this test. In case nitric acid is used, ordinarily 1–2 drops are sufficient.

Repeat the experiment, adding to the first tube 1 drop of acetic acid, to the second 1 drop of concentrated potassium hydroxide solution, to the third 2 drops of a 10 per cent sodium chloride solution and leave the fourth neutral as before.

What is the order of coagulation here? Why? **See page 182.**

12. Precipitation by Alcohol.—Prepare three test-tubes each containing about 10 c.c. of 95 per cent alcohol. To the first add 1 drop of 0.2 per cent hydrochloric acid, to the second 1 drop of potassium hydroxide solution and leave the third neutral in reaction. Add to each tube a few drops of egg albumin solution and note the results. What do you conclude from this experiment?

If in acid or neutral solution alcohol precipitates proteins unaltered, but if allowed to remain under alcohol the protein is transformed. The "fixing" of tissues for histological examination by means of alcohol is an illustration of the application of this transformation produced by alcohol. It apparently is a process of dehydration

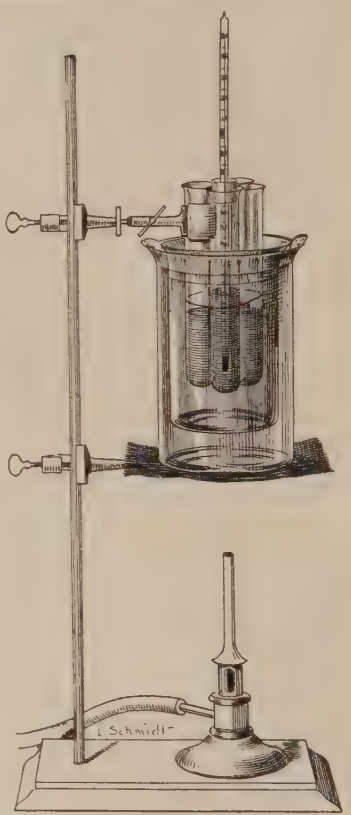


FIG. 49.—COAGULATION TEMPERATURE APPARATUS.

13. Crystallization of Egg Albumin.[1]—Carefully remove the egg-white from a number of absolutely fresh eggs.[2] Measure the volume of the egg-white and add an equal volume of saturated ammonium sulphate a small portion at a time, beating the mixture vigorously after each addition.[3] Filter the mixture through a large pleated filter paper.[4] Measure the volume of the filtrate. To 100 c.c. of the filtrate add very carefully a 10 per cent solution of acetic acid from a burette being certain to note the exact volume of the acid used. The acid should be added drop by drop, the albumin mixture being gently shaken during the process. Add acid until the precipitate which forms at each addition is no longer dissolved when the albumin is shaken, and an opalescent mixture is secured. (It is generally rather difficult to determine this point, inasmuch as suspended air bubbles may simulate a precipitate.) As soon as the solution is milky, indicating that a permanent precipitate has formed, run in from the burette 1 c.c. of the acetic acid. This should produce a heavy white precipitate. Now take the burette reading to determine the exact volume of acid used in the treatment of 100 c.c. of the albumin mixture.

[1] Hopkins and Pinkus: *J. Physiol.*, 23, 130, 1898; 25, 306, 1899. For a slightly different method using sulphuric instead of acetic acid see Sørensen and Hoyrup: *Compt. rend. trav. Lab. Carlsberg*, 12, 12, 1915–17.

[2] If not perfectly fresh the albumin will not crystallize.

[3] Note the odor of ammonia. What causes it?

[4] Sometimes better results are obtained by permitting the mixture to stand several hours before filtering.

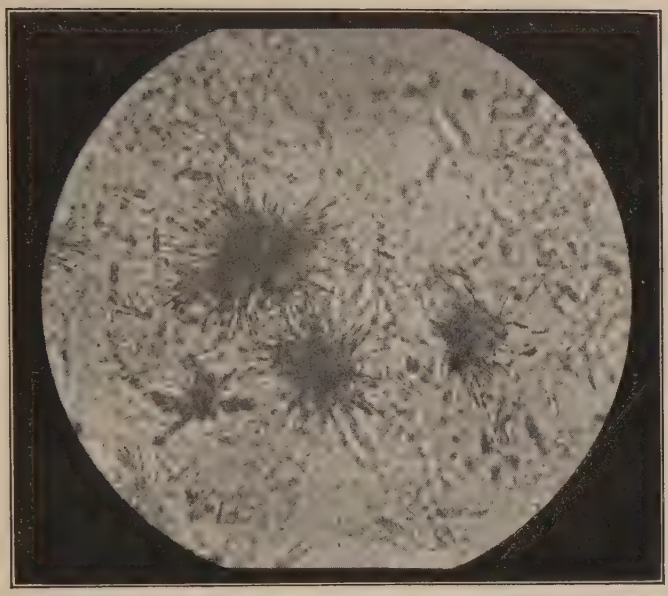


FIG. 50.—CRYSTALLIZED EGG ALBUMIN.

(Prepared and photographed by Mr. Arthur G. Cole, University of Illinois, College of Medicine, Chicago.)

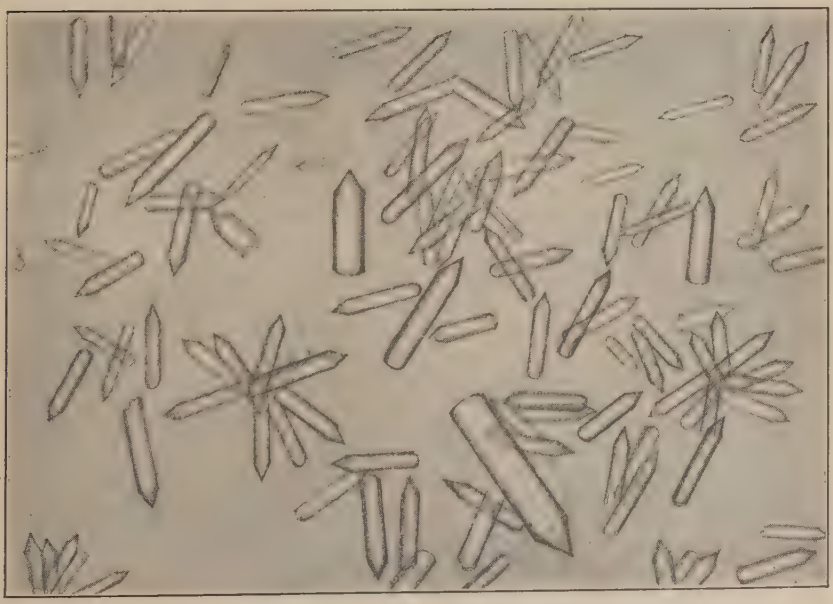


FIG. 51.—HORSE SERUM ALBUMIN.

(Crystals prepared by Professor William H. Welker, University of Illinois, College of Medicine, Chicago.)

Calculate the exact volume of acid necessary to precipitate the remaining portion of the original albumin mixture and add this calculated quantity. Mix the two portions of albumin and allow to stand over night. Remove a drop of the suspended material to a slide and examine microscopically. Crystals in the form of fine needles will be observed. This is the crystallized egg albumin. To recrystallize, filter off the crystals and dissolve them in the smallest possible volume of water. Filter, and to the filtrate carefully add saturated ammonium sulphate until a faint, permanent precipitate is formed. Allow the mixture to stand several hours and examine as before. The crystals of albumin should be somewhat larger than when first examined. For photographs of egg albumin and serum albumin crystals see Figs. 50 and 51.

The above method may also be used for crystallizing serum albumin from the fresh blood serum of the horse, mule or ass.

14. **Preparation of Powdered Egg Albumin.**—This may be prepared as follows: Ordinary egg-white finely divided by means of scissors or a beater is treated with 4 volumes of water and filtered. The filtrate is evaporated on a water-bath at about 50°C. and the residue powdered in a mortar.

15. **Tests on Powdered Egg Albumin.—With powdered albumin prepared as described above (by yourself or furnished by the instructor), try the following tests:**

(a) **Solubility.—Test the solubility of the albumin in water, sodium chloride. dilute acid and alkali.**

(b) **Millon's Reaction.**

(c) **Glyoxylic Acid Reaction (Hopkins-Cole).—When used to detect the presence of protein in solid form this reaction should be conducted as follows: Place 5 c.c. of concentrated sulphuric acid in a test-tube and add carefully, by means of a pipette, 3–5 c.c. of Hopkins-Cole reagent. Introduce a small amount of the solid substance to be tested, agitate the tube slightly, and note that the suspended pieces assume a reddish-violet color, which is the characteristic end-reaction of the Hopkins-Cole test; later the solution will also assume the reddish-violet color.**

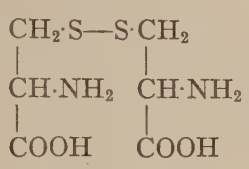
(d) **Composition Test.—Heat some of the dry powder in a dry test-tube in which is suspended a strip of moistened red litmus paper and across the mouth of which is placed a piece of filter paper moistened with lead acetate solution. As the powder is heated it chars, indicating the presence of carbon; the fumes of ammonia are evolved, turning the red litmus paper blue and indicating the presence of nitrogen and hydrogen; the lead acetate paper is blackened, indicating the presence of sulphur, and the deposition of moisture on the side of the tube indicates the presence of hydrogen. Moisture indicates hydrogen only in case both powder and test-tube used in the test are absolutely dry.**

(e) **Coagulation Test.—Immerse a dry test-tube containing a little powdered egg albumin into boiling water for a few moments. Remove and test the solubility of the albumin according to the directions given under (a) above. It is still soluble. Why has it not been coagulated? Repeat the above experiments with powdered serum albumin and see how the results compare with those just obtained.**

SULPHUR IN PROTEIN

Sulphur is believed to be present in two different forms in the protein molecule. The first form, which is present in greatest amount.

is that loosely combined with carbon and hydrogen. An example of this combination is shown in *cystine*,



Sulphur in this form is variously termed *unoxidized, loosely combined, mercaptan*, and *lead-blackening* sulphur. The second form is combined in a more stable manner with carbon and oxygen and is known as *oxidized* or *acid* sulphur. The protamines are the only class of sulphur-free proteins.

Tests for Sulphur

1. Tests for Unoxidized Sulphur.—(a) To equal volumes of KOH and egg albumin solutions in a test-tube add 1–2 drops of lead acetate solution and boil the mixture. Unoxidized sulphur is indicated by a darkening of the solution, the color deepening into a black if sufficient sulphur is present. Add hydrochloric acid and note the characteristic odor evolved from the solution. Write the reactions for this test. (b) Place equal volumes of KOH and egg albumin solutions in a test-tube and boil the mixture vigorously. Cool, make acid with glacial acetic acid and add 1–2 drops of lead acetate. A darkening indicates the presence of unoxidized sulphur.

2. Test for Total Sulphur (Unoxidized and Oxidized).—Place the substance to be examined (powdered egg albumin) in a small porcelain crucible, add a suitable amount of solid fusion mixture (sodium carbonate and potassium nitrate mixed in the proportion 2 : 1) and heat carefully until a colorless mixture results. (Sodium peroxide may be used in place of this fusion mixture if desired.) Cool, dissolve the cake in a little warm water and filter. Acidify the filtrate with hydrochloric acid, heat it to the boiling-point and add a small amount of barium chloride solution. A white precipitate forms if sulphur is present. What is this precipitate?

GLOBULINS

Globulins are simple proteins especially predominant in the vegetable kingdom. They are closely related to the albumins and in common with them give all the ordinary protein tests and are coagulable by heat. Globulins differ from the albumins in being insoluble in pure (salt-free) water. They are, however, soluble in neutral solutions of salts of strong bases with strong acids. Most globulins are precipitated from their solutions by saturation with solid sodium chloride or magnesium sulphate. As a class they are much less stable than the albumins, a fact shown by the increasing difficulty with which a globulin dissolves during the course of successive reprecipitations.

In blood serum there are several forms of globulin characterized by their precipitability by different concentrations of sodium sulphate.[1]

We have used an albumin of animal origin (egg albumin), for all the protein tests thus far, whereas the globulin to be studied will be prepared from a vegetable source. There being no essential difference between animal and vegetable proteins, the vegetable globulin we shall study may be taken as a true type of all globulins, both animal and vegetable.

Experiments on Globulin

Preparation of the Globulin.—Extract 20–30 grams (a handful) of crushed hemp seed with a 5 per cent solution of sodium chloride for one-half hour at 60°C. Filter while hot through a paper moistened with 5 per cent sodium chloride solution. Place the filtrate in a water-bath at 60°C. and allow both to cool spontaneously and stand for 24 hours in order that the globulin may crystallize slowly as the temperature of the bath falls. In case the filtrate is cloudy it should be warmed to 60°C. in order to produce a clear solution. The globulin is soluble

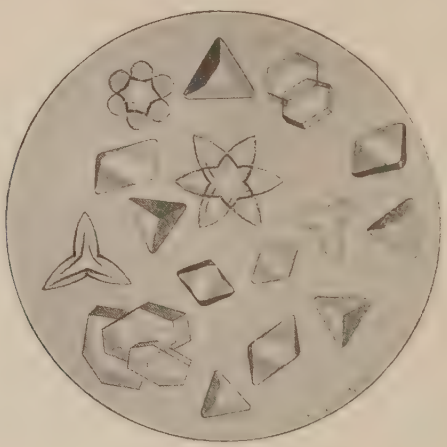


Fig. 52.—Edestin.

in hot 5 per cent sodium chloride solution and is thus extracted from the hemp seed, but upon cooling this solution much of the globulin separates in crystalline form. This particular globulin is called edestin. It crystallizes in several different forms, chiefly octahedra (see Fig. 52, above). (The crystalline form of excelsin, a protein obtained from the Brazil nut, is shown in Fig. 53, p. 175. This vegetable protein crystallizes in the form of hexagonal plates.) Filter off the edestin and make the following tests on the crystalline body and on the filtrate which still contains some of the extracted globulin.

Tests on Crystallized Edestin.—(1) Microscopical examination (see Fig. 52). (2) Solubility.—Try the solubility in the ordinary solvents (see page 71).

[1] Howe, *Jour. Biol. Chem.*, 49, 93, 1921.

(3) Millon's Reaction.

(4) Coagulation Test.—Place a small amount of the globulin in a test-tube, add a little water and boil. Now add dilute hydrochloric acid and note that the protein no longer dissolves. It has been coagulated.

Tests on Edestin Filtrate.—(1) Influence of Protein Precipitants.—Try a few protein precipitants such as nitric acid, tannic acid, picric acid, and mercuric chloride.

(2) Biuret Test.

(3) Coagulation Test.—Boil some of the filtrate in a test-tube. What happens?

(4) Saturation with Sodium Chloride.—Saturate some of the filtrate with solid sodium chloride. How does this result differ from that obtained upon saturating egg albumin solution with solid sodium chloride?

FIG. 53.—EXCELSIN, THE PROTEIN OF THE BRAZIL NUT.
(Drawn from crystals furnished by Dr. Thomas B. Osborne, New Haven, Conn.)

(5) Precipitation by Dilution.—Dilute some of the filtrate with 10–15 volumes of water. Why does the globulin precipitate?

Glutelins

It has been repeatedly shown, particularly by Osborne, that after extracting the seeds of cereals with water, neutral salt solution, and strong alcohol, there still remains a residue which contains protein material which may be extracted by very dilute acid or alkali. These proteins which are insoluble in all neutral solvents, but readily soluble in very dilute acids and alkalis are called *glutelins*. The only member of the group which has yet received a name is the *glutenin* of wheat, a protein which constitutes nearly 50 per cent of the gluten, the remainder being principally *gliadin*. It is not definitely known whether glutelins occur as constituents of all seeds.

Gluten: Preparation and Tests.[1]—To about 50 grams of wheat flour in a casserole or evaporating dish, add a little water and mix thoroughly until a stiff dough results. Knead this dough thoroughly and permit it to stand for about a half hour. This is done in order that the maximum quantity of gluten may be obtained. Treat the dough with about 200 c.c. of water and knead it thoroughly. Note the yellowish color of the dough and the milky appearance of the water due to suspended starch granules. (Place a drop of the suspension on a slide, cover with a cover slip, run underneath the slip a drop of iodine solution and observe the stained starch granules under the microscope.) Filter and apply a protein color reaction (see page 162) to the filtrate. It should be positive, indicating that water-soluble proteins were present in the flour. Add fresh water to the dough and repeat the kneading process. Continue this procedure with fresh addition of water until practically no starch granules are noted in suspension. To a small piece of the yellow, fibrous gluten apply Millon's Reaction (page 162). This test shows gluten to be protein material. Utilize the remainder of the gluten in the preparation of gliadin (page 177).

Glutenin: Preparation and Tests.—(In the preparation of gliadin (page 177) it is customary to remove this prolamin from the crude gluten by extracting with 70 per cent alcohol. Inasmuch as gluten consists chiefly of gliadin and glutenin the portion of the gluten remaining after the extraction of the alcohol-soluble protein gliadin may be utilized for the preparation of glutenin.)

To the finely divided residue from the preparation of gliadin (page 177) in a flask or bottle add about 250 c.c. of 70 per cent alcohol. Allow to stand for about 48 hours with repeated shaking in order to remove any remaining gliadin. Crude glutenin remains. To purify the glutenin treat it in a mortar, with sufficient 0.2 per cent NaOH to dissolve it, and filter the liquid through a wet pleated filter. Neutralize the filtrate carefully, with 0.2 per cent HCl adding the acid drop by drop with thorough mixing after each addition. (The glutenin is soluble in excess of acid.) Filter off the glutenin precipitate and wash several times with 70 per cent alcohol and finally with water. Apply the following tests:

1. Solubility in water, salt solution, 0.2 per cent HCl and 0.5 per cent Na_2CO_3.
2. Millon's Reaction.

Prolamins (Alcohol-soluble Proteins)

The term prolamin has been proposed by Osborne for the group of proteins formerly termed "alcohol-soluble proteins." The name is very appropriate inasmuch as these proteins yield, upon hydrolysis, especially large amounts of *proline* and *ammonia*. The prolamins are simple proteins which are insoluble in water, *absolute* alcohol and other neutral solvents, but are soluble in 70 to 80 per cent alcohol and in dilute acids and alkalis. They occur widely distributed, particularly in the vegetable kingdom. The only prolamins yet described are the *zein* of maize, the *hordein* of barley, the *gliadin* of wheat and rye, the *bynin*

[1] This experiment as well as those on *glutenin* and *gliadin* which follow have been adapted from directions given in laboratory notes of Professor Gies, College of Physicians and Surgeons, New York.

of malt, and the *kafirin* of kafir. Sorghum[1] and rice[2] are also said to contain alcohol-soluble proteins. They yield relatively large amounts of glutamic acid on hydrolysis but *no lysine*. Gliadin of wheat is an exception to this statement containing about 0.6 per cent lysine. The largest percentage of glutamic acid (43.66 per cent) ever obtained as a decomposition product of a protein substance was obtained by Osborne and Guest from the hydrolysis of the prolamin *gliadin*.[3] This yield of glutamic acid is also the largest amount of any single decomposition product yet obtained from any protein *except protamines*.

Gliadin: Preparation and Tests.—Introduce the finely divided crude gluten as prepared on page 176 into a flask or bottle, add about 250 c.c. of 70 per cent alcohol[4] and allow the mixture to stand 24 hours with occasional shaking. Filter (retaining the undissolved portion for preparation of glutenin, page 176), evaporate the filtrate to dryness in a porcelain dish over a water-bath. Pulverize the dry material. Apply the following tests to this gliadin powder:

Solubility and Protein Tests.—Test the solubility in alcohol (30 per cent, 50 per cent and 70 per cent), water, 0.9 per cent NaCl, 0.2 per cent HCl and 0.5 per cent Na_2CO_3. Shake each tube repeatedly and filter. To the filtrate apply Coagulation test (page 169) and Biuret test (page 164).

Albuminoids (Scleroproteins)

The albuminoids yield hydrolytic products similar to those obtained from the other simple proteins already considered, thus indicating that they possess essentially the same chemical structure. They differ from all other proteins, whether simple, conjugated, or derived, in that they are insoluble in all neutral solvents. The albuminoids include "the principal organic constituents of the skeletal structure of animals as well as their external covering and its appendages." Some of the principal albuminoids are *keratin*, *elastin*, *collagen*, *reticulin*, *spongin*, and *fibroin*. Gelatin cannot be classed as an albuminoid although it is a transformation product of collagen. The various albuminoids differ from each other in certain fundamental characteristics which will be considered in detail under Epithelial and Connective Tissue (see Chapter XXIII).

CONJUGATED PROTEINS

Conjugated proteins consist of a protein molecule united to some other molecule or molecules otherwise than as a salt. We have *glyco-*

[1] Hoffman and Gortner: Colloid Symposium Monograph, 1924, ii, Chap. 20.
[2] Hoffman: *Jour. Biol. Chem.*, 66, 51, 1925.
[3] Osborne and Guest: *Jour. Biol. Chem.*, 9, 425, 1911. Up to this time the yield of 41.32 per cent obtained by Kleinschmitt from hordein was the maximum yield.
[4] Bailey and Blish claim that 50 per cent alcohol is more satisfactory (*Jour. Biol. Chem.*, 23, 345, 1915).

proteins, *nucleoproteins*, *hemoglobins* (chromoproteins), *phosphoproteins* and *lecithoproteins* as the five classes of conjugated proteins.

Glycoproteins[1] may be considered as compounds of the protein molecule with a substance or substances containing a carbohydrate group other than a nucleic acid. The *glycoproteins* yield, upon decomposition, protein and carbohydrate derivatives, notably glucosamine, $CH_2OH.(CHOH)_3.CH(NH_2).CHO$, and galactosamine, $CH_2OH.(CHOH)_3.CH(NH_2).CHO$. The principal glycoproteins are *mucoids*, *mucins*, and *chondroproteins*. By the term *mucoid* we may in general designate those glycoproteins which occur in tissues, such as *tendomucoid* from tendinous tissue and *osseomucoid* from bone. (For the preparation of tendomucoid see Chapter XXIII.) The elementary composition of these typical mucoids is as follows:

	N.	S.	C.	H.	O.
Tendomucoid[2]	11.75	2.33	48.76	6.53	30.60
Osseomucoid[3]	12.22	2.32	47.43	6.63	31.40

The term *mucins* may be said in general to include those forms of glycoproteins which occur in the secretions and fluids of the body. (For the preparation of salivary mucin see Chapter IV.) Chondroproteins are so named because *chondromucoid*, the principal member of the group, is derived from cartilage (chondrigen). *Amyloid*,[4] which appears pathologically in the spleen, liver, and kidneys, is also a chondroprotein.

The *phosphoproteins* are considered to be "compounds of the protein molecule and some, as yet undefined, phosphorus-containing substances other than a nucleic acid or lecithin." The percentage of phosphorus in phosphoproteins is very similar to that in nucleoproteins, but they differ from this latter class of proteins in that they do not yield any purine bases upon hydrolytic cleavage. Two of the common phosphoproteins are the *casein* of milk and the *ovovitellin* of the egg-yolk. The phosphorus in these, as in all proteins, exists in phosphoric acid radicals. For the preparation of a typical phosphoprotein (casein) see Chapter XXII.

The *hemoglobins* (chromoproteins) are compounds of the protein molecule with hematin or some similar substance. The principal member of the group is the hemoglobin of the blood. Upon hydrolytic cleavage this hemoglobin yields a protein termed *globin* and a coloring matter termed *hemochromogen*. The latter substance contains *iron* and upon

[1] Levene: Hexosamines and Mucoproteins, Longmans Green and Co., 1925.
[2] Chittenden and Gies: *Jour. Exp. Med.*, 1, 186, 1896.
[3] Hawk and Gies: *Amer. Jour. Physiol.*, 5, 387, 1901.
[4] Not to be confused with the substance *amyloid* which may be formed from cellulose see p. 98).

coming into contact with oxygen is oxidized to form *hematin*. *Hemocyanin*, another member of the class of hemoglobins, occurs in the blood of certain invertebrates, notably cephalopods, gasteropods, and crustacea. Hemocyanin generally contains either *copper*, *manganese*, or *zinc* in place of the *iron* of the hemoglobin molecule. For the preparation of hemoglobin in crystalline form see Chapter XVII.

The *lecithoproteins* consist of a protein molecule joined to lecithin. They have been comparatively little studied and may possibly be *mixtures* of protein and lecithin.

For consideration of *nucleoproteins* see Chapter VII.

DERIVED PROTEINS

These substances are derivatives which are formed through hydrolytic changes of the original protein molecule. They may be divided into two groups, the *primary* protein derivatives and the *secondary* protein derivatives. The term secondary derivatives is made use of in this connection since the formation of the primary derivatives generally precedes the formation of these secondary derivatives. These derived proteins are obtained from native simple proteins by hydrolyses of various kinds, *e.g.*, through the action of acids, alkalis, heat, or enzymes. The particular class of derived protein desired regulates the method of treatment to which the native protein is subjected.

Primary Protein Derivatives

The primary protein derivatives are "apparently formed through hydrolytic changes which involve only slight alterations of the protein molecule." This class includes *proteans*, *metaproteins* and *coagulated proteins*.

PROTEANS

Proteans are those insoluble protein substances which are produced from proteins originally soluble through the incipient action of water, enzymes, or very dilute acids. It is well known that globulins become insoluble upon repeated reprecipitation and it may possibly be found that the greater number of the proteans are transformed globulins. Osborne, however, believes that nearly all proteins may give rise to proteans. This investigator who has so very thoroughly investigated many of the vegetable proteins claims that the hydrogen ion is the active agent in the transformation. The protean produced from the transformation

of *edestin* is called *edestan*, that produced from *myosin* is called *myosan*, etc. The name protean was first given to this class of proteins by Osborne in 1900 in connection with his studies of edestin.

METAPROTEINS

The metaproteins are formed from the native simple proteins through an action similar to that by which proteans are formed. In the case of the *metaproteins*, however, the changes in the original protein molecule are more profound. These derived proteins are characterized by being soluble in very weak acids and alkalis, but *insoluble in neutral fluids*. The metaproteins were formerly termed *albuminates*, but inasmuch as the termination *ate* signifies *a salt* this terminology is inaccurate.

Two of the principal metaproteins are the *acid metaprotein* or so-called acid albuminate and the *alkali metaprotein* or so-called alkali albuminate. They differ from the native simple proteins principally in being insoluble in sodium chloride solution and in not being coagulated *except when suspended in neutral fluids*. Both forms of metaprotein are precipitated upon the approximate neutralization of their solutions. They are precipitated by saturating their solutions with ammonium sulphate, and by sodium chloride also, provided they are dissolved in an acid solution. Acid metaprotein contains a higher percentage of nitrogen and sulphur than the alkali metaprotein from the same source, since some of the nitrogen and sulphur of the original protein is liberated in the formation of the latter. Because of this fact, it is impossible to transform an alkali metaprotein into an acid metaprotein, while it is possible to reverse the process and transform the acid metaprotein into the alkali modification.

Experiments on Metaproteins

ACID METAPROTEIN (ACID ALBUMINATE)

Preparation and Study.—Take 25 grams of hashed lean beef washed free from the major portion of blood and inorganic matter, and place it in a medium-sized beaker with 100 c.c. of 0.2 per cent HCl. Place it on a boiling water-bath for one-half hour, filter, cool, and divide the filtrate into two parts. Neutralize the first part with dilute KOH solution, filter off the precipitate of acid metaprotein and make the following tests:

(1) Solubility.—Solubility in the ordinary solvents (see page 71).

(2) Millon's Reaction.

(3) Coagulation Test.—Suspend a little of the metaprotein in water (neutral solution) and heat to boiling for a few moments. Now add 1–2 drops of KOH solution to the water and see if the metaprotein is still soluble in dilute alkali. What is the result and why?

(4) Test for Unoxidized Sulphur (see page 173).

Subject the second part of the original solution to the following tests:

(5) Coagulation Test.—Heat some of the solution to boiling in a test-tube. Does it coagulate?

(6) Biuret Test.

(7) Influence of Protein Precipitants.—Try a few protein precipitants such as picric acid and mercuric chloride. How do the results obtained compare with those from the experiments on egg albumin? (See page 167.)

ALKALI METAPROTEIN (ALKALI ALBUMINATE)

Preparation and Study.—Carefully separate the white from the yolk of a hen's egg and place the former in an evaporating dish. Add concentrated potassium hydroxide solution, drop by drop, stirring continuously. The mass gradually thickens and finally assumes the consistency of jelly. This is solid alkali metaprotein or "Lieberkühn's jelly." Do not add an excess of potassium hydroxide or the jelly will dissolve. Cut it into small pieces, place a cloth or wire gauze over the dish, and by means of running water wash the pieces free from adherent alkali. Now add a small amount of water, which forms a weak alkaline solution with the alkali within the pieces, and dissolve the jelly by gentle heat. Cool the solution and divide it into two parts. Proceed as follows with the first part: Neutralize with dilute hydrochloric acid, noting the odor of the liberated hydrogen sulphide as the alkali metaprotein precipitates. Filter off the precipitate and test as for acid metaprotein (tests 1, 2, 3 and 4), above, noting particularly the sulphur test. How does this test compare with that given by the acid metaprotein? Make tests on the second part of the solution the same as for acid metaprotein (tests 5, 6 and 7) above.

Coagulated Proteins

These derived proteins are produced from unaltered protein materials by heat, by long standing under alcohol, or by the continuous movement of their solutions such as that produced by rapid stirring or shaking. In particular instances, such as the formation of fibrin from fibrinogen (see page 341), the coagulation may be produced by enzyme action. Ordinary soluble proteins after having been transformed into the coagulated modification are no longer soluble in the ordinary solvents. Upon being heated in the presence of strong acids or alkalis, coagulated proteins are converted into metaproteins.

Many proteins coagulate at an approximately fixed temperature under definite conditions (see Exp. 11 page 169). This characteristic may be applied to separate different coagulable proteins from the same solution by fractional coagulation. The coagulation temperature frequently may serve in a measure to identify proteins in a manner similar to the melting-point or boiling-point of many other organic substances.

The separation of proteins by fractional coagulation is thus analogous to the separation of volatile substances by means of *fractional distillation*. This method of separating proteins is not a satisfactory one, however, inasmuch as proteins in solution have different effects upon one another and also because of the fact that the nature of the solvent causes a variation in the temperature at which a given protein coagulates. The nature of the process involved in the coagulation of proteins by heat is not well understood. According to Sørensen[1] the reaction is not one of hydrolysis, the protein actually losing water. Others however believe the underlying process to be hydrolytic in character.[2] The presence of a neutral salt or a trace of mineral acid may facilitate the coagulation of a protein solution (see page 169), whereas any appreciable amount of acid or alkali will retard or entirely prevent such coagulation.

It has been shown that the coagulation of proteins by heat proceeds in two stages:[3] first, denaturation of the protein and second, an agglutination or separation of the altered protein in particulate form. The concentration of acid, or hydrogen ion, in the solution influences the coagulation of proteins, so that the original protein is acted upon less readily in hot water alone than in the presence of acid. The formation of the coagulum is accompanied by the disappearance of the free acid from the solution, indicating the formation of a protein salt. A disturbance of the equilibrium between the hydrolyzed and unhydrolyzed portions of the protein salt, due to the greater rapidity with which the unhydrolyzed portion is precipitated, results in the gradual removal of both protein and acid from the solution. This has been offered as an explanation of the decreasing acidity.

According to Chick and Martin, the addition of neutral salts to the acid solution of the salt-free protein to be coagulated results in a *decreased rate* of coagulation. This is due in part to the decrease in the concentration of the free acid, which results from the disturbance of the equilibrium between the protein and acid and also in part to the direct influence which the salts exert upon the protein. The presence of neutral salts may under certain circumstances facilitate the coagulation of proteins by heat.

The temperature at which egg-white is coagulated causes a difference in the appearance of the coagulum.[4] Coagulated egg-white which has been immersed in water at a *low* temperature and then gradually heated to the coagulating temperature is more translucent and has a bluish

[1] Sørensen: Proteins, The Fleischmann Laboratories, New York, 1925.
[2] Wu and Wu: *Jour. Biol. Chem.*, 64, 369, 1925.
[3] **Chick and Martin**. *Journal of Physiology*, **43, 1, 1911.**
[4] Frank: *Journal of Biological Chemistry*, 9, 463, 1911.

color, whereas egg-white which has been immersed in water heated to a temperature *above* the coagulating temperature is creamy white in color. They also possess different digestibilities.

Experiments on Coagulated Protein

Ordinary coagulated egg-white may be used in the following tests:

1. Solubility.—Try the solubility of small pieces of coagulated protein in each of the ordinary solvents (see page 71).

2. Millon's Reaction.

3. Xanthoproteic Reaction.—Partly dissolve a medium-sized piece of the protein in concentrated nitric acid. Cool the solution and add an excess of ammonium hydroxide. Both the protein solution and the undissolved protein will be colored orange.

4. Biuret Test.—Partly dissolve a medium-sized piece of the protein in concentrated potassium hydroxide solution. If the proper dilution of copper sulphate solution is now added the white coagulated protein, as well as the protein solution, will assume the characteristic purplish-violet color.

5. Glyoxylic Acid Reaction (Hopkins-Cole).—Conduct this test according to the modification given on page 172.

Secondary Protein Derivatives

These derivatives result from a more profound cleavage of the protein molecule than that which occurs in the formation of the primary derivatives. The class included *proteoses*, *peptones*, and *peptides*.

PROTEOSES AND PEPTONES

Proteoses are intermediate products in the digestion of proteins by proteolytic enzymes, as well as in the decomposition of proteins by hydrolysis and the putrefaction of proteins through the action of bacteria. Proteoses are called *albumoses* by some writers, but it seems more logical to reserve the term albumose for the proteose of albumin.

Peptones are formed after the proteoses and it has been customary to consider them as the last product of the processes before mentioned which still possess true protein characteristics. In other words, it has been considered that the protein nature of the end-products of the cleavage of the protein molecule ceased with the peptones, and that the simpler bodies formed from peptones were substances of a different nature (see page 115). However, as the end-products have been more carefully studied, it has been found to be no easy matter to designate the exact character of a peptone or to indicate the exact point at which the *peptone* characteristic ends and the *peptide* characteristic begins. The situation regarding the proteoses, peptones and peptides

is at present a most unsatisfactory one because of the unsettled state of our knowledge regarding them. The exact differences between certain members of the peptone and peptide groups remain to be more accurately established. It has been quite well established that the peptones are peptides or mixtures of peptides, but the term peptide is used at present to designate only those possessing a definite structure.

There are several proteoses (protoproteose, heteroproteose and deuteroproteose), and at least two peptones (amphopeptone and antipeptone), which result from proteolysis. The differentiation of the various proteoses and peptones at present in use is rather unsatisfactory. These compounds are classified according to their varying solubilities, especially in ammonium sulphate solutions of different strengths. The exact differences in composition between the various members of the group remain to be more accurately established. Because of the difficulty attending the separation of these bodies, pure proteose and peptone are not easy to procure. The so-called peptones sold commerically contain a large amount of proteose. As a class the proteoses and peptones are very soluble bodies not coagulated by heat. The peptones are diffusible whereas most of the proteoses are not. *Peptones differ from proteoses in being more diffusible, non-precipitable by* $(NH_4)_2SO_4$, *and by their failure to give any reaction with potassium ferrocyanide and acetic acid, potassio-mercuric iodide and* HCl, *picric acid*, and *trichloracetic acid*. Peptones may be precipitated by phosphotungstic acid, phosphomolybdic acid, absolute alcohol and tannic acid, but an excess of the precipitant may dissolve the precipitate. The so-called *primary proteoses* are precipitated by HNO_3 and are the only members of the proteose-peptone group which are so precipitated.

Some of the more general characteristics of the proteose-peptone group may be noted by making the following simple tests on a proteose-peptone powder:

(1) Solubility.—Solubility in hot and cold water and sodium chloride solution.

(2) Millon's Reaction.

Dissolve a little of the powder in water and test the solution as follows:

(3) Precipitation by Picric Acid.—To 5 c.c. of proteose-peptone solution in a test-tube add picric acid until a permanent precipitate forms. The precipitate disappears on heating and returns on cooling.

(4) Precipitation by a Mineral Acid.—Try the precipitation by nitric acid.

(5) Coagulation Test.—Heat a little proteose-peptone solution to boiling. Does it coagulate like the other simple proteins studied?

SEPARATION OF PROTEOSES AND PEPTONES[1]

Place 50 c.c. of proteose-peptone solution in an evaporating dish or casserole, and half-saturate it with ammonium sulphate solution, which may be accom-

[1] The separation of proteoses and peptones by means of fractional precipitation with ammonium sulphate does not possess the significance it was once supposed to possess inasmuch as the boundary between these substances and *peptides* is not well defined (see p. 183).

plished by adding an equal volume of saturated ammonium sulphate solution. At this point note the appearance of a precipitate of the primary proteoses (protoproteose and heteroproteose). Now heat the half-saturated solution and its suspended precipitate to boiling and saturate the solution with solid ammonium sulphate. At full saturation the secondary proteoses (deuteroproteoses) are precipitated. The peptones remain in solution.

Proceed as follows with the precipitate of proteoses: Collect the sticky precipitate on a rubber-tipped stirring rod or remove it by means of a watch glass to a small evaporating dish and dissolve it in a little water. To remove the ammonium sulphate, which adhered to the precipitate and is now in solution, add barium carbonate, boil, and filter off the precipitate of barium sulphate. Concentrate the proteose solution to a small volume[1] and make the following tests:

(1) Biuret Test.

(2) Precipitation by Nitric Acid.—What would a precipitate at this point indicate?

(3) Precipitation by Trichloracetic Acid.—This precipitate dissolves on heating and returns on cooling.

(4) Precipitation by Picric Acid.—This precipitate also disappears on heating and returns on cooling.

(5) Precipitation by Potassio-mercuric Iodide and Hydrochloric Acid.

(6) Coagulation Test.—Boil a little in a test-tube. Does it coagulate?

(7) Acetic Acid and Potassium Ferrocyanide Test.—The solution containing the peptones should be cooled and filtered, and the ammonium sulphate in solution removed by boiling with barium carbonate as described above. After filtering off the barium sulphate precipitate, concentrate the peptone filtrate to a small volume and repeat the tests as given under the proteose solution, above. Also try the precipitation by phosphotungstic acid and by tannic acid. In the biuret test the solution should be made very strongly alkaline with solid potassium hydroxide.

PEPTIDES

The peptides are "definitely characterized combinations of two or more amino acids, the carboxyl (COOH) group of one being united with the amino (NH_2) group of the other with the elimination of a molecule of water." These peptides are more fully discussed on pages 121 and 183.

REVIEW OF PROTEINS

In order to facilitate the student's review of the proteins, the preparation of a chart similar to the model given is recommended. The signs + and − may be conveniently used to indicate positive and negative reactions.

[1] If the proteoses are desired in powder form, this concentrated proteose solution may now be precipitated by alcohol, and this precipitate, after being washed with absolute alcohol and with ether, may be dried and powdered.

MODEL CHART FOR REVIEW PURPOSES

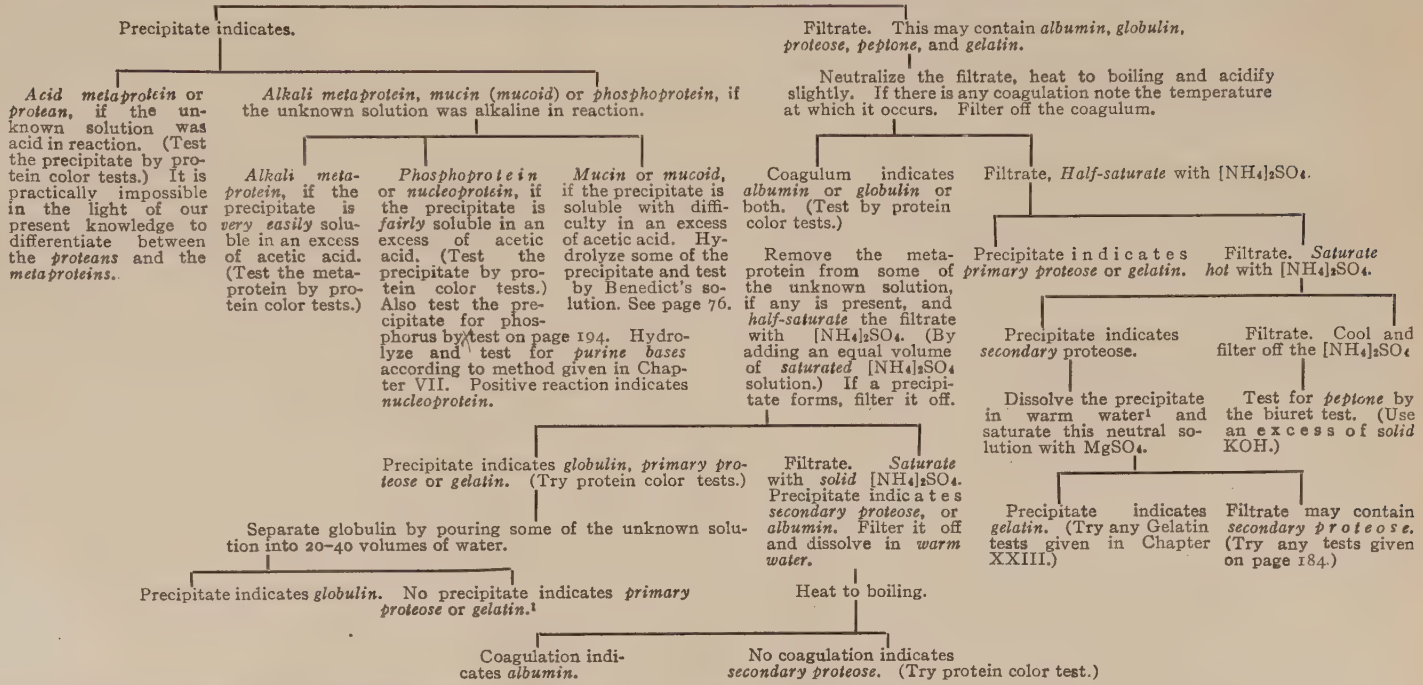
Protein	Solubility						Precipitation Tests							Salting-out Tests		Diffusion	Coagulation by Heat
	Water	10 % NaCl	0.2 % HCl	0.5 % Na_2CO_3	Conc. HCl	Conc. KOH	Protein Color Test	Mineral Acid (HNO_3)	Metallic Salt ($HgCl_2$)	Alcohol	Pot. Ferrocyanide + Acetic Acid	Potassio-mercuric Iodide + HCl	Picric Acid	Trichloracetic Acid	$(NH_4)_2SO_4$	NaCl		
Albumin																	
Globulin																	
Nucleoprotein																	
Phosphoprotein																	
Glycoprotein																	
Acid metaprotein																	
Alkali metaprotein																	
Proteose																	
Peptone																	
Coagulated protein																	

"Unknown" Mixtures and Solutions of Proteins

At this point the student's knowledge of the characteristics of the various proteins studied will be tested by requiring him to examine several "unknown" protein mixtures or solutions and make full report upon the same. The scheme given on page 187 may be used in this examination.

SCHEME FOR THE DETECTION OF PROTEINS

If the solution is acid or alkaline it should be *approximately* neutralized.
If a precipitate forms it should be filtered off. The neutralization need not necessarily proceed until an exact neutral reaction is obtained but should cease at the point where the largest precipitate is secured.


Precipitate indicates.
Filtrate. This may contain *albumin*, *globulin*, *proteose*, *peptone*, and *gelatin*.
Acid metaprotein or *protean*, if the unknown solution was acid in reaction. (Test the precipitate by protein color tests.) It is practically impossible in the light of our present knowledge to differentiate between the *proteans* and the *metaproteins*.
Alkali metaprotein, *mucin* (*mucoid*) or *phosphoprotein*, if the unknown solution was alkaline in reaction.
Neutralize the filtrate, heat to boiling and acidify slightly. If there is any coagulation note the temperature at which it occurs. Filter off the coagulum.
Alkali metaprotein, if the precipitate is *very easily* soluble in an excess of acetic acid. (Test the metaprotein by protein color tests.)
Phosphoprotein or *nucleoprotein*, if the precipitate is *fairly* soluble in an excess of acetic acid. (Test the precipitate by protein color tests.) Also test the precipitate for phosphorus by test on page 194. Hydrolyze and test for *purine bases* according to method given in Chapter VII. Positive reaction indicates *nucleoprotein*.
Mucin or *mucoid*, if the precipitate is soluble with difficulty in an excess of acetic acid. Hydrolyze some of the precipitate and test by Benedict's solution. See page 76.
Coagulum indicates *albumin* or *globulin* or both. (Test by protein color tests.)
Filtrate, *Half-saturate* with $[NH_4]_2SO_4$.
Remove the metaprotein from some of the unknown solution, if any is present, and *half-saturate* the filtrate with $[NH_4]_2SO_4$. (By adding an equal volume of *saturated* $[NH_4]_2SO_4$ solution.) If a precipitate forms, filter it off.
Precipitate indicates *primary proteose* or *gelatin*.
Filtrate. *Saturate hot* with $[NH_4]_2SO_4$.
Precipitate indicates *secondary* proteose.
Filtrate. Cool and filter off the $[NH_4]_2SO_4$
Dissolve the precipitate in warm water[1] and saturate this neutral solution with $MgSO_4$.
Test for *peptone* by the biuret test. (Use an excess of *solid* KOH.)
Precipitate indicates *gelatin*. (Try any Gelatin tests given in Chapter XXIII.)
Filtrate may contain *secondary proteose*. (Try any tests given on page 184.)
Precipitate indicates *globulin*, *primary proteose* or *gelatin*. (Try protein color tests.)
Filtrate. *Saturate* with *solid* $[NH_4]_2SO_4$. Precipitate indicates *secondary proteose*, or *albumin*. Filter it off and dissolve in *warm water*.
Separate globulin by pouring some of the unknown solution into 20–40 volumes of water.
Precipitate indicates *globulin*. No precipitate indicates *primary proteose* or *gelatin*.[1]
Heat to boiling.
Coagulation indicates *albumin*.
No coagulation indicates *secondary proteose*. (Try protein color test.)

[1] We may also differentiate between gelatin and proteose by means of the Hopkins-Cole reaction (see page 163). A positive reaction here would indicate proteose and a negative reaction would indicate gelatin.

CHAPTER VII

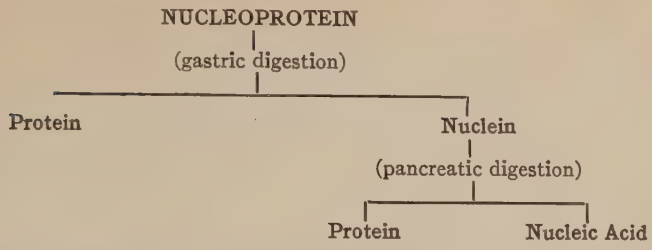
NUCLEIC ACIDS AND NUCLEOPROTEINS[1]

The Nucleoproteins.—The nucleoproteins occur widely distributed in the animal and plant kingdoms, being found in nearly all cells and particularly in the nuclei of cells. They are found in especially large amounts in glandular tissues such as those of the thymus, pancreas and spleen. The nucleoproteins are combinations of protein with a phosphorus-containing substance known as nucleic acid. As different nucleic acids exist and are found in combination with different proteins, a variety of nucleoproteins exist. The protein combined with the nucleic acid is in certain cases a histone, the conjugated protein in this case being called a nucleohistone.

The nucleoproteins give the ordinary protein color reactions. They are acidic in character and insoluble in water. They are readily soluble in weak alkali but are precipitated from such solution on the addition of acetic acid in excess of which they dissolve with more or less difficulty although readily soluble in excess of dilute hydrochloric acid. We distinguish them from mucins, which are likewise precipitated by acetic acid through the fact that the latter give no tests for phosphorus on decomposition.

The nucleoproteins are very complex and unstable substances and are probably in many cases to be considered as mixtures of protein and nucleic acid rather than as definite compounds. Under the action of the gastric juice or of weak acid nucleoproteins lose a portion of their protein content and are transformed into a rather ill-defined class of substances known as *nucleins* which still possess some protein in combination with the nucleic acid molecule. In most cases the decomposition does not proceed further in gastric digestion. Through the action of the pancreatic juice, however, the remainder of the protein is split off and the nucleic acid set free. The decomposition of nucleoprotein may be diagrammatically expressed thus, although the course of decomposition is probably not quite so simple as indicated.

[1] For review of the literature on nucleic acids and nucleases see Monograph on "Nucleic Acids" by Walter Jones. New York, 1924, Longmans Green & Co.



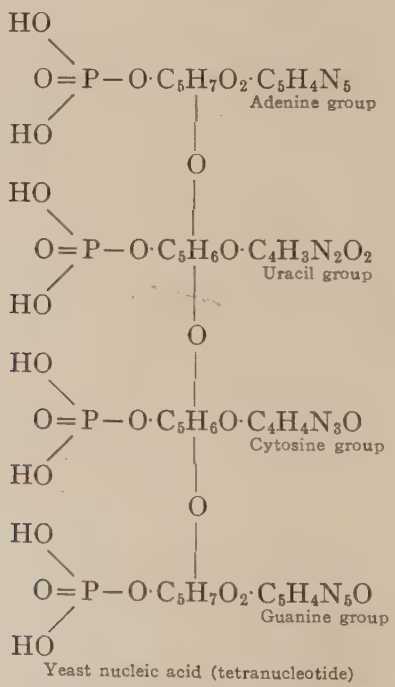
The Nucleic Acids.—The nucleic acids of the animal body occur mainly in combination with protein material in the so-called nucleoproteins of which they form the characteristic radicals (see page 190). The amount and character of the protein with which the nucleic acid molecule is combined varies and the acid may in certain cases be found in cells in a free form. Naturally those tissues are richest in nucleic acid which contain the largest amount of nuclear material and of nucleoprotein. Such are the glandular tissues of the body as the thymus, spleen, pancreas, liver, etc. The heads of the spermatozoa consist almost entirely of nucleic acid in combination with protamine.

The nucleic acids are a distinct class of substances, characterized by their decomposition products. They are strongly acid in reaction and contain considerable phosphorus. They may be divided into two main groups, the *animal* and the *plant* nucleic acids. The two classes differ in certain respects but all of the true animal nucleic acids appear to be practically identical in composition. Animal nucleic acid is most readily prepared from the thymus while plant nucleic acid is most readily obtained from yeast.

The nucleic acids are difficultly soluble in cold water, more readily in hot water, insoluble in alcohol, but readily soluble in weak alkali with the formation of the alkali salt. If pure they do not give the protein color reactions. They are optically active. They are precipitated from their alkaline solutions by HCl, but only the plant nucleic acid is precipitated by acetic acid. In weak acid solution they are precipitated by protein the combination being considered a "nuclein." They form insoluble salts with alkaline earth and heavy metals. The sodium salt of animal nucleic acid in 4 per cent solution is liquid while warm but solidifies to a gelatinous mass on cooling. Plant nucleic acid does not do this.

The nucleic acids on hydrolysis yield phosphoric acid, purine and pyrimidine bases, and a carbohydrate or carbohydrate derivative. The composition varies slightly with the type of nucleic acid. Plant nucleic acids contain a pentose group (*d*-ribose) while animal nucleic acids contain a hexose group. Both types contain the purine bases, guanine and adenine and the pyrimidine base cytosine. Plant nucleic acid contains

also the pyrimidine base uracil, which in the animal nucleic acid is substituted by the base thymine. The nucleic acids are not, however, simple substances whose molecules contain a single phosphoric acid or carbohydrate group. They are apparently combinations of several radicals known as *nucleotides* each of which contains one carbohydrate group combined with a single base and a single phosphoric acid molecule. Thus the following structural formula was suggested for yeast nucleic acid by Jones and Read[1] indicating that it contains four nucleotide radicals linked through the carbohydrate groups. Jones and Perkins have presented evidence[2] favoring a structure containing one ether linkage of this type and two ester (phosphoric acid to carbohydrate) linkages. Levene holds[3] that all three linkages are of the ester type. Opinions differ as to whether uracil exists in the molecule as such or is a decomposition product of cytosine[4].



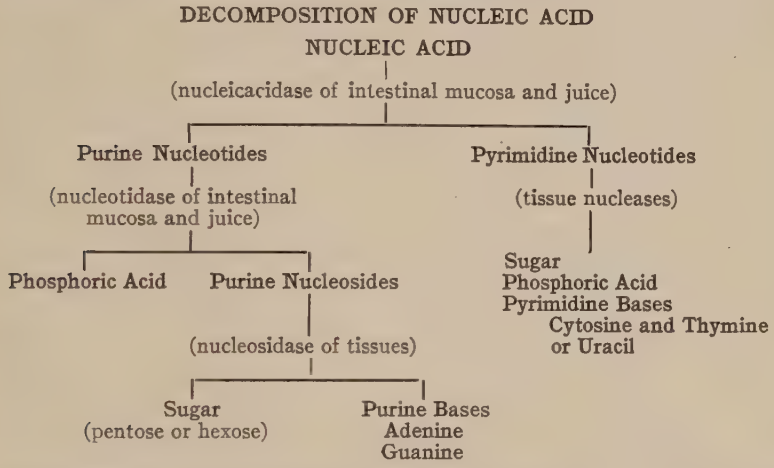
Yeast nucleic acid (tetranucleotide)

The cleavage of the nucleic acid molecule into its corresponding nucleotides is brought about during digestion by enzymes present in the intestinal juice and intestinal mucosa. Enzymes of similar origin act further on the nucleotides thus formed and split off the phosphoric

[1] Jones and Read: *Jour. Biol. Chem.*, 31, 111, 1917.
[2] Jones and Perkins: *Jour. Biol. Chem.*, 55, 557 and 567, 1923.
[3] Levene and Simms: *Jour. Biol. Chem.*, 65, 519, 1925.
[4] Jones and Perkins: *Jour. Biol. Chem.*, 62, 557, 1925; Levene: *Jour. Biol. Chem.*, 67, 325, 1926.

acid radicals together with carbohydrate-base compounds which are called *nucleosides*. The decomposition prior to absorption does not probably proceed further than to the formation of nucleotides and nucleosides. Many tissues however contain enzymes capable of completing the decomposition with liberation of the carbohydrate and basic radicals.[1] The purine bases may also be deaminized while still in combination as nucleosides and further hydrolysis would then lead to the direct liberation of the oxypurines instead of their precursors, the amino-purines.

The following outline will indicate the course of decomposition of a nucleic acid and the enzymes involved in the process.



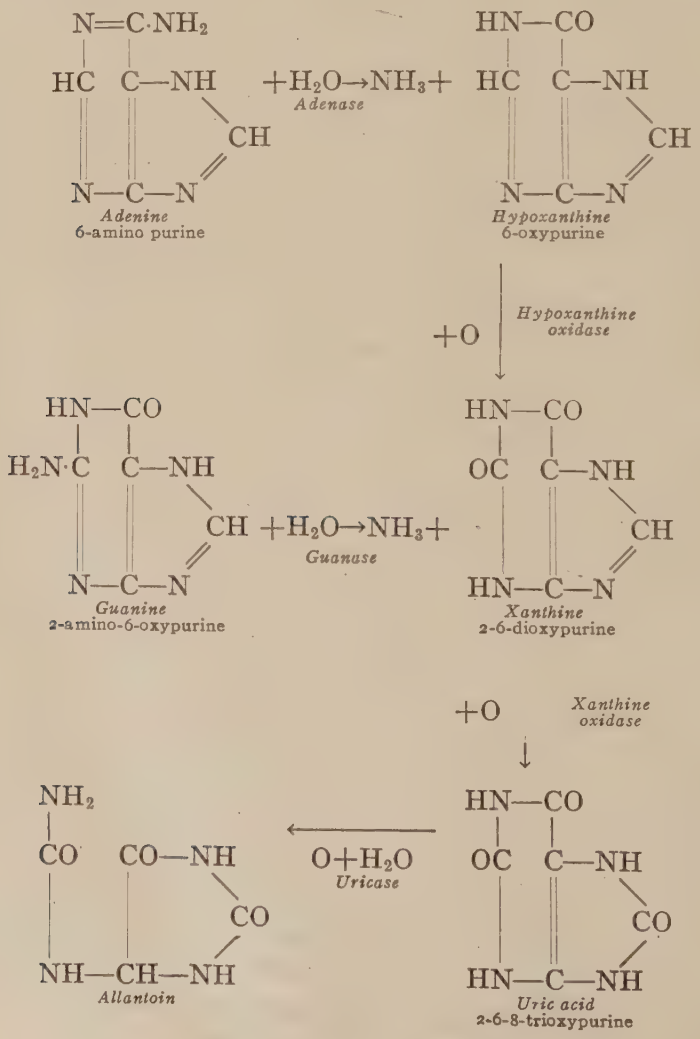
With regard to the fate of the various radicals of the nucleic acids in the body after absorption little is definitely known. The phosphoric acid may of course be built up into phosphorus-containing cell constituents such as nucleoproteins, phosphoproteins or phosphatides, or be eliminated as phosphate in the urine. The carbohydrate portion may undergo the usual transformations of intermediary carbohydrate metabolism. The nucleosides appear to be ordinarily absorbed unchanged from the intestine and may be to a certain extent directly resynthesized in the animal body to nucleoprotein. The excess over body requirement must, however, be decomposed, although a certain portion may possibly be stored up in the individual cells or in certain organs. Enzymes capable of decomposing nucleic acids are found in most of the cells of the body.

The Purine Bases.[2]—As has been indicated the basic substances present in nucleic acid belong to two classes the *purine* and *pyrimidine*

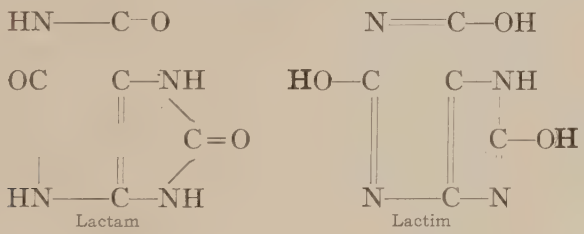
[1] For preparation and properties of nucleosidase see Levene, Yamagawa and Weber: *Jour. Biol. Chem.*, 60, 693, 1924.

[2] For review of purine metabolism see Rose: *Physiol. Reviews*, 3, 544, 1923.

bases. The purine bases set free on the decomposition of nucleic acid are *adenine and guanine* belonging to the class of *amino purines*. The fate of the amino purines in the animal body is of considerable interest. It has been shown that certain tissues contain enzymes which transform these amino purines first to corresponding *oxypurines* known as *hypoxanthine and xanthine* and finally to *uric acid*. It is probable that different enzymes enter into the various steps of these transformations leading to the formation of uric acid. Still another enzyme carries the oxidation further with the formation of the compound *allantoin*. This enzyme is known as uricase. The purine enzymes are widely distributed in tissues. The transformations brought about are indicated in the following scheme.

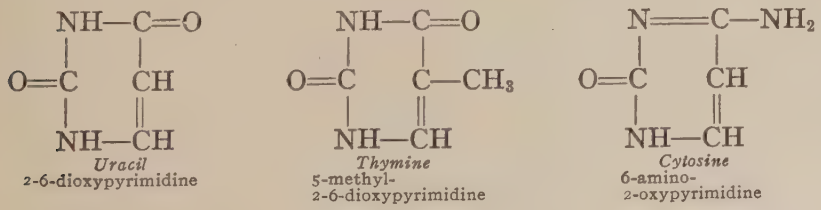


All of the physiologically important purine bodies are precipitated by ammoniacal silver nitrate solution in the cold and by copper sulphate and sodium bisulphite in boiling solutions. Some of them are readily identified by their crystalline forms or the crystalline forms of certain of their salts. Uric acid differs from the other purines in being insoluble in dilute sulphuric acid. The purine bodies may be distinguished to a certain extent also by the reactions which they give when their solutions are evaporated with nitric acid and the residue treated with ammonia. Uric acid gives the characteristic formation of the purple murexide (ammonium purpurate). Potassium hydroxide changes this to a bluish-violet color which disappears on heating. Xanthine and guanine form yellow compounds with nitric acid which turn purple or violet on treating with potassium hydroxide. The color in this case is not lost by heating. Adenine and hypoxanthine do not give a color reaction with nitric acid.



The keto-enol tautomerism which uric acid is known to possess, is illustrated in the above formulas. The lactam form is less stable. Two series of salts of the lactim form result from substitution of the hydrogen atoms indicated in black face type. The third enolic hydrogen is probably very slightly dissociated.

The Pyrimidine Bases.—The pyrimidine bases entering into the composition of nucleic acid are *thymine*, *cytosine* and *uracil*. Cytosine is found in both types of nucleic acid, while thymine is found only in animal nucleic acid and uracil only in plant nucleic acid. They possess the following formulas.



With regard to the fate of pyrimidine bases in metabolism very little is known. When the bases as such are fed they reappear

unchanged in the urine.[1] If nucleic acid is fed this does not occur which indicates that the pyrimidine bases may undergo certain alterations in the animal body while still existing in combination. It has been suggested that these bases or their derivatives may have a respiratory function.[2]

Experiments

1. Preparation of Nucleoprotein from Yeast.[3]—Place two small cakes of ordinary compressed yeast in a mortar. Sprinkle a small horn-spoonful of sand over the yeast, add 5 c.c. of ether and 10 c.c. of water and thoroughly triturate the mixture, grinding vigorously. The ether kills the yeast, in which condition the comminution of the cells with sand is more thoroughly effected. Occasionally during the trituration process add 1 or 2 c.c. of water until the mixture is comparatively fluid. The whole process of maceration can be completed in five minutes. Pour the thick liquid into a bottle aiding the transfer with enough 0.4 per cent NaOH to make a final volume of about 125 c.c. The alkali extracts the nucleoprotein along with the water-soluble proteins of the yeast. Add a little toluene and allow to stand with frequent shaking for 12–24 hours. Filter through a wet, fluted filter. While thoroughly stirring add 1 drop at a time of 10 per cent HCl cautiously continuing the addition as long as the milkiness of the mixture can be increased. Continue until the protein completely separates and the liquid is practically clear. Note that the solution is now acid in reaction. Excess of acid causes resolution. Filter on a wet, fluted filter. Retain the precipitate on the filter for nucleoprotein tests.

2. Tests on Nucleoprotein.—Try the following tests on the nucleoprotein prepared as above.

(a) Try the xanthoproteic and Millon's tests.

(b) Test the solubility in water, 10 per cent NaCl, 10 per cent HCl, dilute KOH, and alcohol.

(c) Tests for Phosphorus in Organic Matter.—Test for organically combined phosphorus by one of the following methods. (1) Fusion Test.—To a small amount of the substance in a crucible add about five times its bulk of fusion mixture (2 parts of sodium carbonate to 1 of potassium nitrate). Heat carefully until the resulting mixture is colorless. Cool, dissolve the mass in a little warm water, acidify with nitric acid, heat to about 65°C. and add a few cubic centimeters of molybdate solution. In the presence of phosphorus a yellow precipitate of ammonium phosphomolybdate is formed.

Instead of acidifying with nitric acid, the aqueous solution may be approximately neutralized with hydrochloric acid, a few cubic centimeters of magnesia mixture added and then excess of ammonium hydroxide solution. A white precipitate of magnesium ammonium phosphate is formed.

(2) Moist Ashing Procedure.—Treat a small amount of the substance in a large test-tube with about 1 c.c. of concentrated sulphuric acid. Then add drop by drop an equal volume of concentrated nitric acid, and warm gently until a clear solution is obtained. A few more drops of nitric acid may be added if necessary.

[1] Mendel and Myers: *Am. J. Physiol.*, 26, 77, 1910.
[2] Baudisch: *Jour. Biol. Chem.*, 60, 155, 1924.
[3] All experiments on nucleoprotein of yeast have been taken from laboratory notes of Professor W. J. Gies, of College of Physicians and Surgeons, New York.

This treatment with sulphuric and nitric acids must be carried out with the greatest caution particularly when fatty substances are present; otherwise an explosive reaction may take place. Dilute the acid solution with a little water, make slightly alkaline with ammonia and then acid with nitric acid. Add molybdate solution and warm. A yellow precipitate is formed.

(d) Dissolve a little of the precipitate in very dilute KOH and then make slightly acid with acetic acid.

(e) Mix a small portion of the nucleoprotein with 10 c.c. of alcohol. Filter and wash free from HCl with more alcohol. (Freedom from HCl is indicated by absence of $AgNO_3$-chloride reaction in the filtrate.) Wash free from alcohol with a little water. Transfer small particles of the precipitate to moistened red and blue litmus paper on a microscopic slide. What is the reaction of nucleoprotein thus freed from adherent acid?

3. To Show the Presence of Purine Base Radicals in Nucleoprotein.—The nucleic acid portion of the protein molecule contains phosphoric acid, carbohydrate, and purin base radicals (see page 191). Hence on the complete acid hydrolysis of nucleoprotein material these substances will be liberated as well as the decomposition products of the protein part of the molecule. To show their presence proceed as follows: Transfer the precipitate of nucleoprotein remaining from the previous experiment to a small flask and add 25–50 c.c. of 5 per cent H_2SO_4. Boil for an hour or more to decompose. Maintain the original volume by adding water. The solution becomes brown due to formation of melanin-like substances. The purine bases are set free. Retain one-fourth of the solution for the next experiment. Transfer the remainder to a casserole and add ammonia with thorough mixing, a little at a time, until the fluid is nearly neutral. Then make slightly alkaline with dilute ammonia and filter if not clear. Transfer to a beaker and add about 10 c.c. of 5 per cent ammoniacal silver nitrate solution.[1] Purine bases if present will yield a brown flocculent precipitate of their silver compounds. If a precipitate does not appear immediately, examine the solution after it has been allowed to stand for some time undisturbed.

4. To show the Presence of Protein, Carbohydrate, and Phosphoric Acid Radicals in Nucleoprotein.—Filter the greater portion of the acid liquid which was reserved from the preceding experiment. Apply the following tests to portions of it: (a) The biuret test. (b) The xanthoproteic test. (c) Molisch test. (d) Benedict's test. (e) Test for phosphate.

5. Preparation of Thymus Nucleoprotein.—About 100 grams of fresh thymus gland (lymphatic glands may also be used) freed as nearly as possible from adherent fat are run through a meat chopper. To this material in a flask add 300 c.c. of 0.9 per cent NaCl and allow to stand 24–48 hours in the cold. A little chloroform and toluene should be added as preservatives, and the mixture shaken occasionally during this period. Filter. A milk white liquid is obtained. Precipitate the nucleoprotein from solution by the careful addition of dilute acetic acid. Excess of the acid should be avoided. Ordinarily acetic acid to make a 1 per cent solution is sufficient. Filter off the precipitate. Wash with alcohol and then with ether and dry.

6. Experiments on Thymus Nucleoprotein.—Repeat the experiments given under Yeast Nucleoprotein (page 194).

7. Preparation of Yeast Nucleic Acid.—Dilute 50 c.c. of 1 per cent NaOH with 250 c.c. of water in a casserole and add to this solution 100 grams of compressed yeast cut in small pieces. Heat on the water-bath for half an hour with

[1] See Reagents and Solutions (Appendix).

occasional stirring. Remove from the bath and filter at once through a folded filter. To the cooled filtrate add acetic acid until faintly acid to litmus. Filter again. Evaporate the solution to 100 c.c. or less and filter if necessary. Allow to cool to 40°C. or below, then pour with vigorous stirring into 200 c.c. of 95 per cent alcohol containing 2 c.c. of concentrated HCl. Allow to settle and wash the precipitate by decantation in a tall vessel, twice with 95 per cent alcohol and twice with ether. Transfer to a filter paper. Allow to drain and dry at room temperature.

8. Tests on Nucleic Acid from Yeast.[1]—1. Test the solubility of nucleic acid in cold and hot water, in alcohol, and in dilute acid and alkali. To the solution in alkali add dilute HCl drop by drop until the solution is acid, then add excess of concentrated HCl.

Does nucleic acid coagulate on boiling? Does the solution in hot water gelatinize on cooling?

2. Try xanthoproteic reaction and biuret test.

3. Dissolve a little nucleic acid in water with the aid of heat. Test the reaction of different portions of the solution with litmus, alizarin, and Congo red solution.

4. Boil a small portion of the nucleic acid with about 10 c.c. of 10 per cent sulphuric acid for one to two minutes. Divide into three portions.

(a) To one portion apply carbohydrate tests, e.g., the α-naphthol (Molisch) reaction and Bial's test. What do these indicate?

(b) To a second portion apply a test for purine bases. Add an excess of ammonia and then a little silver nitrate solution.

(c) To the third portion apply test for phosphate, adding ammonia in slight excess, then making acid with nitric acid, adding molybdic solution and warming.

9. Preparation of Animal Nucleic Acid.[2]—Dissect 250 grams of thymus, pancreas, kidney, or spleen free from fat and grind in a chopping machine. Transfer to a casserole containing 300 c.c. of 5 per cent sodium hydroxide. Boil for thirty-five minutes. Neutralize with acetic acid. Add 50 c.c. of colloidal iron solution (Merck's 5 per cent). Filter and allow to stand over night. To the filtrate add two volumes of methyl alcohol, containing 2 per cent of hydrochloric acid. Filter and wash the precipitate with methyl alcohol until free from hydrochloric acid.

10. Tests on Animal Nucleic Acid.—1–4. Repeat the experiments as given under yeast nucleic acid, above. 5. Make a 4 per cent solution of thymus nucleic acid in hot water (0.4 gram to 10 c.c.). Allow to cool. What happens? Divide into two portions. To one add a little NaOH solution; to the other add acetic acid. Then neutralize carefully in each case.

Both acetic acid and NaOH decrease the viscosity of the nucleate solution. It may be changed back and forth from the gelatinous to the fluid condition by the alternate addition of acid and alkali.

11. Tests on Purine Bases and Derivatives.—(a) Xanthine.—(1) Silver Nitrate Reaction.—Dissolve a little xanthine in ammonia and add silver nitrate solution. Examine a little of the precipitate microscopically.

(2) Copper Sulphate Reaction.—Dissolve a little of the substance in dilute alkali, make faintly acid with acetic acid. Heat to boiling. Add 1 c.c. 10

[1] A satisfactory preparation of yeast nucleic acid may be obtained from Merck and Co.
[2] Levene: *Jour. Biol. Chem.*, 53, 441, 1922.

per cent $CuSO_4$ and then a few drops at a time of sodium bisulphite (saturated solution) until the precipitate becomes yellowish. All of the purines give this reaction.

(3) Nitric Acid Test.—Place a small amount of the substance in a small evaporating dish, add a few drops of concentrated nitric acid, and evaporate to dryness very carefully on a water-bath. The yellow residue upon moistening with caustic potash becomes red in color and upon further heating assumes a purplish-red hue. Now add a few drops of water and warm. A yellow solution results which yields a red residue upon evaporation. Compare with similar reaction on other purine bases and uric acid. (See Murexide test, Chapter XXVII.)

(4) Weidel's Reaction.—Bring a small amount of the substance into solution in bromine water. Evaporate to dryness on a water-bath. Remove the stopper from an ammonia bottle and by blowing across the mouth of the bottle direct the

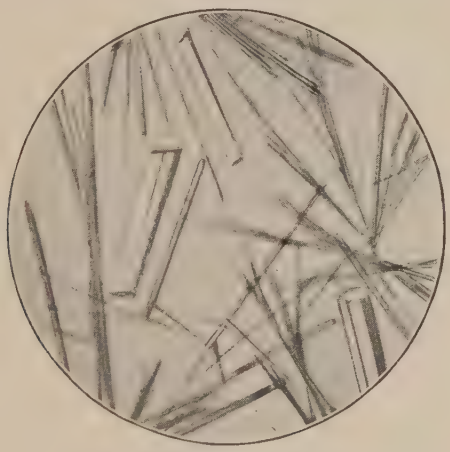


FIG. 54.—GUANINE CHLORIDE.
(Reproduced from crystals furnished by Professor Walter Jones.)

fumes of ammonia so that they come into contact with the dry residue. Under these conditions the presence of xanthine is shown by the residue assuming a red color. A somewhat brighter color may be obtained by using a trace of nitric acid with the bromine water. By the use of this modification, however, we may get a positive reaction with bodies other than xanthine.

(b) Hypoxanthine.—(1) Repeat Experiments (1) and (3) under Xanthine. Examine the crystals of hypoxanthine silver nitrate under the microscope. (See Fig. 176.)

(2) Dissolve a little of the substance in a very small amount of hot 6 per cent nitric acid and allow to cool. Characteristic whetstone crystals of hypoxanthine nitrate should be formed. Examine under the microscope. (See Fig. 177.)

(c) Adenine.—(1) Warm a few crystals of adenine in a test-tube with a little water. They should become cloudy at 53°C.

(2) Dissolve a little adenine in hot water and add a few drops of picric acid. Examine the pale yellow crystals under the microscope. The picrate crystallizes as needle clusters.

(3) Repeat Experiment (3) under Xanthine.

(d) Guanine.—(1) Dissolve a little substance in 20–25 times its weight of boiling 5 per cent alcohol. Allow to cool and examine crystals microscopically.

(2) For other tests on uric acid see Chapter XXVII on Urine.

(3) Dissolve a little guanine in 20–25 times its weight of boiling 5 per cent hydrochloric acid. Allow to cool and examine crystals under microscope. (See Fig. 54.)

(4) Perform Experiment 3 under Xanthine.

(e) Uric Acid.—1. On a small amount of uric acid try the test as given under Xanthine (3). This test on uric acid is called the Murexide test.

12. Isolation of Guanine and Adenine from Nucleic Acid (Method of Walter Jones).[1]—The amino purines may be isolated from yeast or thymus nucleic acid or from glandular tissue (such as the pancreas) after hydrolysis of the material with sulphuric acid.

Place 5 g. of yeast nucleic acid in a 200 c.c. Erlenmeyer flask, add 100 c.c. of boiling hot 5 per cent sulphuric acid and heat carefully over a small flame to bring the nucleic acid into solution. During this heating which lasts only a minute, the contents of the flask should be kept constantly in motion to avoid burning the undissolved nucleic acid. Close the flask with a soft cork, bored with one hole into which is inserted a condensing tube, immerse in a boiling water bath so that the surface of the liquid in the flask is not below the level of the water in the water bath and allow to heat for about an hour. Disconnect the flask and while its contents are still hot, add concentrated ammonia drop at a time until the fluid is roughly alkaline and then add 5 c.c. of concentrated ammonia in excess. Guanine is thus precipitated in granular form and can be easily filtered off, while all of the other products including phosphoric acid and adenine remain in the ammoniacal solution.

After several hours filter off the precipitate of guanine and wash it with 1 per cent ammonia. Dissolve in as small an amount of 20 per cent sulphuric acid as possible, add a little animal charcoal and boil. Filter, heat to boiling and precipitate with excess of ammonia. Filter, dry the precipitate at 40°C. and dissolve it in about 20 parts of boiling 5 per cent hydrochloric acid. As the solution cools guanine chloride separates out as needle-shaped crystals. (See Fig. 54, p. 197). Filter, off wash with very dilute hydrochloric acid and dry in the air (do not put in desiccator). Perform the nitric acid test on the product.

Combine the ammoniacal filtrates obtained in the isolation and purification of guanine. Filter if necessary. The ammonia may then be boiled off and an excess of picric acid added in which case a yellow precipitate of adenine picrate is produced which is filtered off and dried. It is better, however, to neutralize the ammonia of the combined filtrates and make faintly acid with sulphuric acid. Then precipitate the adenine as its copper compound decomposing this with hydrogen sulphide and evaporating the filtrate from the copper sulphide to dryness on the water-bath. Dissolve the residue in hot 5 per cent sulphuric acid and allow to crystallize out. If necessary dissolve in hot water decolorize with a little charcoal and allow to crystallize out again. The compound has the formula $(C_5H_5N_5)_2.H_2SO_4.2H_2O$. Apply the picric acid and nitric acid tests as given under adenine (page 197).

[1] See Walter Jones: Monograph on "Nucleic Acids," 1924, Longmans, Green & Co. Levene: (*Jour. Biol. Chem.*, 48, 177, 1921) decomposes by suspending 10 gm. of nucleic acid in 200 c.c. of dry methyl alcohol and passing a lively stream of hydrochloric acid gas through the mixture for two hours and then allowing to stand over night.

13. The Pyrimidine Derivatives.—The pyrimidine derivatives, cytosine, thymine, and uracil, are separated from nucleic acid with some difficulty. The following test may be made on a solution of cytosine or uracil. Thymine does not give the test.

Wheeler-Johnson Reaction for Uracil and Cytosine.—To about 5 c.c. of the solution under examination add bromine water until the color is permanent. Avoid the addition of a large excess as this will interfere with the test. In case the solution contains only small quantities of cytosine or uracil it is advisable to remove any excess of bromine by passing a stream of air through the solution. Now add an excess of an aqueous solution of barium hydroxide and note the appearance of a purple color.

Very dilute solutions do not give the test. Under these conditions the solution should be evaporated to dryness, the residue dissolved in a little bromine water and the excess of bromine removed. Then upon adding an excess of barium hydroxide a decided bluish-pink or lavender color will appear in the presence of as small an amount as 0.001 gram of uracil.

In testing solutions for cytosine it is preferable to warm or boil the solution with bromine water, and after cooling the solution to apply the test as suggested above, being careful to have a slight excess of bromine present before adding barium hydroxide.

CHAPTER VIII

GASTRIC DIGESTION

Gastric digestion takes place in the stomach and is promoted by the gastric juice, which is secreted by the glands of the stomach mucosa. These glands are of two kinds, fundus glands and pyloric glands which are situated, as their names imply, in the regions of the fundus and pylorus. The principal foods acted upon in gastric digestion are the proteins which are so changed by its processes as to become better prepared for further digestion in the intestine and for their final absorption.

From reliable experiments made upon lower animals and man it is evident that the gastric juice is secreted as the result of stimuli of two forms, *i.e.*, *psychical* stimuli and *chemical* stimuli.[1] The psychical form of stimuli may be produced by the sight, thought, or taste of food, and the chemical stimuli may be produced by certain substances, such as water, milk, the extractives of meat, etc., when coming in contact with the stomach mucosa. The stimulatory power of water has been very strikingly demonstrated.[2] The claim that the drinking of water with meals is harmful because such a procedure causes a dilution of the gastric juice, has no basis in fact. The drinking of water with meals by normal individuals has been found to be accompanied by a more economical utilization of the ingested proteins, fats and carbohydrates. Various other desirable and *no undesirable* features have been demonstrated as accompanying or following such a dietary procedure.[3] No *experimental* evidence has been submitted which can justly be interpreted as showing any harmful influence to accompany or follow the drinking, by normal persons, of large quantities of water at meal time.[4]

[1] Other factors may also be concerned. See next paragraph.

[2] Foster and Lambert: *Journ. Exper. Med.*, 10, 820, 1908.
Bergeim, Rehfuss and Hawk: *Jour. Biol. Chem.*, 19, 345, 1914.
Ivy: *Proceedings Amer. Physiol. Soc.* (*Amer. Jour. Physiol.*) 45, 561, 1918.
King and Hanford: *ibid.*, 562.
Sutherland: *ibid.*, 563.

[3] Hawk: *University of Pennsylvania Medical Bulletin*, 18, 1, 1905.
Fowler and Hawk: *Jour. Exper. Med.*, 12, 388, 1910.
Hattrem and Hawk: *Arch. Int. Med.*, 7, 610, 1911.
Mattill and Hawk: *Jour. Am. Chem. Soc.*, 33, pp. 1978, 1999, and 2019, 1911.
Hawk: *Arch. Int. Med.*, 8, 382, 1911.
Hawk: *Proceedings Soc. Exp. Biol. and Med.*, 8, 36, 1910.
Fairhall and Hawk: *Jour. Am. Chem. Soc.*, 34, 546, 1912.
Howe and Hawk: *Jour. Biol. Chem.*, 11, 129, 1912.
Hawk: *Biochem. Bull.*, 3, 420, 1914.

[4] For a discussion of "Water as a Dietary Constituent" see Hawk: *Endocrinology and Metabolism*, (Barker, Hoskins and Mosenthal), Vol. 3, p. 275, Appletons, 1924.

The subcutaneous injection of histamine also stimulates gastric secretion.[1] Gastric pouches severed from the stomach and transplanted elsewhere in the body (region of mammary gland) of the dog have been found to secrete gastric juice after a meal and also after the subcutaneous injection of histamin[2] and gastrin.[3] It seems, therefore, that a *humoral stimulus* is one of the mechanisms concerned in the genesis of gastric secretion. Mechanical distension by a balloon caused a secretion of gastric juice in these pouches, thus indicating that a *local mechanism* is also concerned.[4]

The volume of gastric juice secreted during any given period of digestion varies with the quantity and kind of the food. These conclusions were deduced principally from a series of so-called *delusive feeding* experiments. A dog was prepared with two esophageal openings and a gastric fistula. When thus prepared and fed foods of various kinds such as meat and bread, the material instead of passing to the stomach, would invariably find its way out of the animal's body at the upper esophageal opening. Through the medium of the gastric fistula the course of the secretion of gastric juice could be carefully followed. It was found that when the dog ate meat, for example, there was a large secretion of gastric juice notwithstanding no portion of the food eaten had reached the stomach. Further experiments made through the medium of a *cul-de-sac* formed from the stomach wall have given us many valuable conclusions, among others those regarding the influence of the chemical stimuli. The method followed was to feed the animal certain substances and note the secretion of gastric juice in the miniature stomach while the real process of digestion was taking place in the stomach proper.

Normal gastric juice is a thin, light colored fluid which is acid in reaction and has a specific gravity varying between 1.001 and 1.010. It contains only 0.5 per cent of solid matter which is made up principally of sodium chlorid, potassium chloride, earthy phosphates, mucin and the enzymes *pepsin*, *gastric rennin*, and *gastric lipase;* the enzymes are of the greatest importance. The acidity of the gastric juice is due to *free* hydrochloric acid. It was formerly believed that this acid was secreted by the parietal cells of the fundus as well as by the chief cells of both the fundus and pyloric glands. It has been claimed,[5] however, that *the parietal cell is the seat of the formation of the hydrochloric acid.* This

[1] Gompertz and Vorhaus, *Jour. Lab. Clin. Med.*, 11, 14, 1925.
[2] See p. 292.
[3] A basic substance extracted from the gastric mucosa (Keeton and Koch): *Am. Jour. Physiol.*, 37, 481, 1915.
[4] Ivy and Farrell: *Am. Jour. Physiol.*, 74, 639, 1925.
[5] Fitzgerald: *Proceedings Royal Society* (B), 83, 56, 1910.

conclusion is based upon the formation of Prussian blue after the subcutaneous injection of potassium ferrocyanide and ammonium ferric citrate (rabbits and guinea-pigs) and the subsequent (3 to 30 hours) microscopical examination of the gastric mucosa. The acid was shown to be present in the lumina of the gland tubules and in the canaliculi of the parietal cells; traces were also apparently present in the cytoplasm. Later Bensley and Harvey[1] showed by means of dyes (cyanamin) which act as vital stains and as indicators very sensitive to alkali, that the *secretion in the parietal cells is slightly alkaline whereas that in the lumen of the gland proper is very nearly neutral.* Therefore, they say, the acid is formed entirely above the level of the gland proper, *i.e.*, in the foveolæ and on the surface. Hammet,[2] and Macallum and Collip[3] confirmed Miss Fitzgerald's claim that the acid is formed in the parietal cells. Dawson and Ivy[4] have demonstrated that the canaliculi and cytoplasm of the active parietal cell were alkaline to cyanamin and acid to neutral red (pH 3.0 to pH 6.8).

It was believed that hydrochloric acid was generally present in the gastric juice of man to the extent of about 0.2 per cent. When the amount of hydrochloric acid varied to any considerable degree from this value a condition of hypoacidity or hyperacidity was said to be established. On the basis of more recent experiments,[5] however, it appears that the actual acidity of the gastric juice of man as secreted by the glands is 0.4 to 0.5 per cent hydrochloric acid. Boldyreff believes that this acidity is lowered to about 0.2 per cent by regurgitation of alkaline fluid from the intestine (Chapter IX on Gastric Analysis). Hydrochloric acid has the power of combining with protein substances taken in the food, thus forming so-called *combined* hydrochloric acid. This combined acid is a less potent germicide than *free* hydrochloric acid and has less power to destroy the amylolytic enzyme *salivary amylase* (*ptyalin*) of the saliva. This last fact explains to a degree the possibility of the continuance of salivary digestion in the stomach.

The term *combined* hydrochloric acid is really a misnomer. When *free* hydrochloric acid is treated with a protein the latter functions as a base and a *salt* is formed. Therefore, instead of having "*combined hydrochloric acid*" we have a *protein salt of hydrochloric acid.* This salt ionizes differently from the free acid. This fact explains the variation in the germicidal properties of the two solutions as well as

[1] Bensley and Harvey: *Biological Bulletin*, 23, 225, 1912.
[2] Hammett: *Anatomical Record*, 9, 21, 1915.
[3] Reported before Society of Biological Chemists, Boston, Dec. 27, 1915.
[4] Dawson and Ivy: *Am. Jour. Physiol.*, 76, 158, 1926.
[5] Babkin: Die äussere Sekretion der Verdauungsdrüsen, Berlin, 1914, p. 113.
Boldyreff: *Quart. Jour. Exper. Physiol.*, 8, 1, 1914.
Carlson: *Am. Jour. Physiol.*, 38, 248, 1915.

their different action toward enzymes, such, for example, as salivary amylase (see page 107).

The hydrochloric acid of the gastric juice forms a medium in which the pepsin can most satisfactorily digest the protein food, and at the same time it acts as an antiseptic or germicide which prevents putrefactive processes in the stomach. It also possesses slight power of inverting cane sugar, this property being due to the hydrogen ion. When the hydrochloric acid of the gastric juice is diminished in quantity (hypoacidity) or absent, as it may be in many cases of functional or organic disease, there is no check to the growth of micro-organisms in the stomach. There are, however, certain of the more resistant spores which even the normal acidity of the gastric juice will not destroy. A condition of hypoacidity may also give rise to fermentation with the formation of comparatively large amounts of such substances as lactic acid and butyric acid.

The question of the origin of the hydrochloric acid of the gastric juice is a problem to whose solution many investigators have given much attention. Many theories have been proposed, among them the interaction of sodium chloride with carbonic acid,[1] with acid phosphate[2] or with organic acids. On the other hand, it is possible that hydrochloric acid is set free from combination with some weak base as ammonia[3] or that free phosphoric acid valences may be set free by the enzymic hydrolysis of organic phosphoric acid esters.[4] The presence of another acid-forming enzyme in the gastric mucosa has also been suggested.[5] We cannot go into a discussion of these various theories. That the hydrochloric acid arises from the chlorides of the blood appears to be well established but the same cannot be said with regard to the immediate or ultimate origin of the hydrogen ions involved in the reaction.

The most important of the enzymes of the gastric juice is the proteolytic enzyme *pepsin*. The pepsin does not originate as such in the gastric cells but is formed from its precursor, the *zymogen* or mother-substance *pepsinogen*, which is apparently produced by the parietal cells of the fundus as well as by the chief cells of the fundus and pyloric glands. Pepsinogen may be differentiated from pepsin from the fact that it is more resistant to alkali.[6] Upon coming into contact with the

[1] Bunge: Physiologic and Pathologic Chemistry, 2nd. Eng. Ed., Philadelphia, 1902, p. 135.

[2] Maly: *Zeit. f. physiol. Chem.*, 1, 174, 1877. Macallum and Collip: Reported before the Society of Biological Chemists, Boston, Dec. 27, 1915.

[3] Mathews: Physiological Chemistry, Fourth Edition, New York, 1925, p. 386.

[4] Bergeim: Proc. Soc. Exp. Biol. and Med., 12, 21, 1914.

[5] Hanke: *Jour. Biol. Chem.*, 67, xi, 1926.

[6] Langley: *Jour. of Physiol.*, 3, 246, 1880–82.

hydrochloric acid of the secretion this pepsinogen is immediately transformed into pepsin. Pepsin is not active in alkaline or neutral solutions, the optimum hydrogen ion concentration being between pH 2 and 3. At about pH 5 its power to digest protein disappears. The percentage of hydrochloric acid facilitating the most rapid peptic action varies with the character of the protein acted upon, *e.g.*, 0.08 per cent to 0.1 per cent for the digestion of fibrin and 0.25 per cent for the digestion of coagulated egg-white. While hydrochloric acid is the acid usually employed to promote artificial peptic proteolysis, other acids, organic and inorganic, will serve the same purpose. Acidity of the liquid is necessary to promote the activity of the pepsin, but the acidity need not necessarily be confined to hydrochloric acid.

In common with many other enzymes pepsin acts best at about 38°–40°C. and its digestive power decreases as the temperature is lowered, the enzyme being only slightly active at 0°C. Its power is only temporarily inhibited by the application of such low temperatures, however, and the enzyme regains its full proteolytic power upon raising the temperature to 40°C. As the temperature of a digestive mixture is raised above 40°C. the pepsin gradually loses its activity until at about 80°–100°C. its proteolytic power is permanently destroyed. It is also inactivated by ultraviolet radiation.[1]

Our ideas regarding the nature of the products formed in the course of peptic proteolysis have undergone considerable revision in recent years. The former view that these products included only acid albuminate (acid metaprotein), proteoses, peptones and peptides is no longer tenable. From the investigations of numerous observers we have learned that artificial gastric digestion *if permitted to proceed for a sufficiently long period* will yield, in addition to proteoses, peptones and peptides, a long list of protein cleavage products which are crystalline in character, including *leucine*, *tyrosine*, *alanine*, *phenylalanine*, *aspartic acid*, *glutamic acid*, *proline*, *leucinimide*, *valine*, and *lysine*. A similar group of substances may result from the action of the enzyme trypsin (see page 256). The relative amounts of proteoses, peptones, and crystalline substances formed depends to a great extent upon the character of the protein undergoing digestion, *e.g.*, a greater proportion of proteoses results from the digestion of fibrin than from the digestion of coagulated egg-white. We must not be led into the error of thinking that the large number of protein cleavage products just mentioned are formed in the course of normal gastric digestion *within the animal organism*. They are formed only after comparatively long-continued

[1] Hussey and Thompson: *Jour. Gen Physiol.*, 9, 217, 1925. For a discussion of Ultraviolet Radiation, see Chapter XXXII.

hydrolysis. In pancreatic digestion, however, there are formed even under normal conditions the large number of cleavage products to which reference has been made. Peptic proteolysis, therefore, within the animal organism differs from tryptic proteolysis (see page 256) in that the former yields larger amounts of proteoses, smaller amounts of peptones and no considerable quantity of crystalline bodies as end-products in the brief period during which proteins are ordinarily subjected to gastric digestion. Prolonged hydrolysis with gastric juice does, however, yield considerable quantities of the non-protein end-products. In cases of cancer of the stomach a peptide-splitting enzyme (erepsin) is said to be present in the stomach contents. This enzyme is believed to be elaborated by the cancer tissue and its identification is of importance in connection with the diagnosis of gastric cancer. The glycyl-trytophane test[1] is sometimes used for this purpose. This test has been very severely criticized (see page 270).

Abderhalden and Meyer[2] have shown active pepsin to be present in the contents of all parts of the small intestine. It is suggested that pepsin may be adsorbed in the stomach by such protein substances as pass into the intestine in solid form and that the pepsin thus protected may bring about gastric digestion whenever the reaction of the surrounding intestinal contents is favorable. This fact may be of importance in connection with the profound proteolysis taking place in the intestine. Heretofore, this process was believed to be furthered alone by trypsin and erepsin. The passage of adsorbed pepsin into the intestine may be an efficient aid to the proper digestion of solid proteins which are ingested without sufficient mastication ("bolted")[3] and which consequently, at times, pass into the intestine in rather large pieces (see Chapter XVI on Feces).

Gastric rennin, the second enzyme of the gastric juice, is what is known as a *milk curdling* or *protein coagulating* enzyme. Rennin acts upon the casein of the milk, splitting it into a *peptone-like* body and *soluble paracasein*. This soluble body, in the presence of calcium salts, combines with calcium, forming *calcium paracaseinate* which is insoluble and precipitates. There is some uncertainty regarding the reaction in which gastric rennin shows the greatest activity. It is, however, said to be active in neutral, alkaline, or acid solution. However, it probably possesses its greatest activity in the presence of a slight acid reaction, as would naturally be expected. It is especially abundant in the gastric mucosa of the calf, and is used to curdle the

[1] Neubauer and Fischer: *Deut. Arch. f. klin. Med.*, 97, 499, 1909.
[2] Abderhalden and Meyer: *Zeit. für physiol. Chem.*, 74, 67, 1911.
[3] Foster and Hawk: *Proceedings of the Eighth International Congress of Applied Chemistry*, New York, September, 1912.

milk used in cheese making. Gastric rennin is always present normally in the gastric juice, but in certain pathological conditions such as atrophy of the mucosa, chronic catarrh of the stomach, or in carcinoma it may be absent.

The theory that the proteolytic activity and the milk curdling property of the gastric juice reside in a single substance, has been the subject of much controversy. The theory was originally advanced by the Pawlow school.[1] According to Nencki and Sieber,[2] the milk curdling and protein hydrolyzing activities reside in definite and distinct side chains of a single mammoth molecule. The view which has rather the strongest support, however, is to the effect that there are two entirely distinct enzymes. Important evidence has been advanced in favor of this view of Hammarsten,[3] Taylor,[4] and Hemmeter.[5] Burge[6] has reported experiments upon the influence of a direct electric current upon solutions possessing typical rennin and peptic activities. By this means he was able to prepare a solution possessing strong rennin activity but entirely devoid of peptic activity. This furnishes strong evidence against the identity of the two enzymes but does not necessarily deny the accuracy of the side-chain theory.

Gastric lipase, the third enzyme of the gastric juice, is a fat-splitting enzyme. It possesses but slight activity when the gastric juice is of normal acidity, but evinces its action principally at such times as a gastric juice of low acidity is secreted either from physiological or pathological cause. The digestion of fat in the stomach is, however, at most, of but slight importance as compared with the digestion of fat in the intestine through the action of the lipase of the pancreatic juice (see page 258).

Boldyreff[7] has shown *trypsin* to be present in stomach contents, due to regurgitation of intestinal contents through the pylorus. This claim has been verified by others[8] (see Chapter IX on Gastric Analysis).

THE NORMAL GASTRIC RESPONSE TO STANDARD FOODS

On the basis of extensive studies made in the senior author's laboratory[9] the following tables have been constructed which contain

[1] Pawlow and Parastschuk: *Zeitschrift für Physiologische Chemie*, 42, 415, 1904.
[2] Nencki and Sieber: *Zeitschrift für Physiologische Chemie*, 23, 191, 1901.
[3] Hammarsten: *Zeitschrift für Physiologische Chemie*, 56, 18, 1908; 94, 291, 1915.
[4] Taylor: *Journal of Biological Chemistry*, 5, 399, 1909.
[5] Hemmeter: *Berliner klinische Wochenschrift*, Ewald Festnummer, 44, 1905.
[6] Burge: *American Journal of Physiology*, 29, 1912.
[7] Boldyreff: *Quart. Jour. Exper. Physiol.*, 8, 1, 1914.
[8] Spencer Meyer, Rehfuss and Hawk: *Am. Jour. Physiol.*, 39, 459, 1916.
[9] I. Intragastric Conductance—Bergeim: *Am. J. Physiol.*, 45, 1, 1917.
II. Milk—Bergeim, Evvard, Rehfuss, Hawk: *Am. J. Physiol.*, 48, 411, 1919.
III. Beef—Fishback, Smith, Bergeim, Lichtenthaeler, Rehfuss, Hawk; *Am. J. Physiol.*, 49, 174, 1919.
IV. Pork—Smith, Fishback, Bergeim, Rehfuss, Hawk: *Am. J. Physiol.*, 49, 204, 1919.
V. Lamb—Fishback, Smith, Bergeim, Rehfuss, Hawk: *Am. J. Physiol.*, 49, 222, 1919.
VI. Eggs—Miller, Fowler, Bergeim, Rehfuss, Hawk: *Am. J. Physiol.*, 49, 254, 1919.

EVACUATION TIMES FOR VARIOUS ARTICLES OF DIET

ARTICLES OF DIET 100-gram portions unless otherwise stated	NUMBER OF OBSERVATIONS	EVACUATION TIME (hours and minutes)		
		Type of stomach		
		Rapid emptying	Slow emptying	Average
Beef and beef products	25	2.35	3.25	3.00
Bread and cereals[1]	75	....	....	2.40
Cakes	29	....	....	3.00
Chicken[1]	20	2.45	3.45	3.15
Egg and egg combinations	90	2.15	3.15	2.40
Fish[1]	75	....	....	2.50
Fruits[1]	68	1.35	2.20	2.00
Gelatin[1]	5	....	....	2.00
Guinea hen[1]	2	....	4.00	4.00
Ice cream[1]	7	....	....	3.15
Ices[1]	4	....	....	2.35
Junket	4	....	....	2.25
Lamb and lamb products	14	2.30	3.20	3.00
Licorice	1	....	....	3.00
Milk:[1]				
Cows':				
400 c.c.	50	....	....	2.30
75 c.c.	3	....	....	1.15
Mothers':				
150 c.c.	5	....	....	1.40
225 c.c.	2	....	....	2.25
Nuts:[1]				
(*a*) 25 gm	18	....	....	3.00
(*b*) 50 gm	4	....	....	4.00
Orange albumin (2:1)	2	....	....	2.20
Pies	29	....	....	2.30
Pop corn	3	....	....	1.30
Pork and pork products	31	2.45	3.40	3.15
Puddings	23	....	....	2.20
Sugars and candies	28	....	....	2.05
Turkey[1]	2	3.00	3.45	3.30
Veal:		....	....	
(*a*) Market	7	....	....	2.50
(*b*) "Bob"[1]	7	....	....	3.20
Vegetables prepared in different ways	124	2.00	2.30	2.15

VII. Vegetables—Miller, Fowler, Bergeim, Rehfuss, Hawk: *Am. J. Physiol.*, 51, 332 1920.

VIII. Unpalatable Food—Holder, Smith, Hawk: *Sci.*, 51, 299, 1920.

IX. Influence of Worry—Miller, Bergeim, Hawk: *Sci.*, 52, 253, 1920.

X. Psychic Secretion—Miller, Bergeim, Rehfuss, Hawk: *Am. J. Physiol.*, 52, 1, 1920.

XI. Tea, Coffee, Cocoa—Miller, Bergeim, Rehfuss, Hawk: *Am. J. Physiol.*, 52, 28, 1920.

XII. Pies, Cakes, Puddings—Miller, Fowler, Bergeim, Rehfuss, Hawk: *Am. J. Physiol.*, 52, 248, 1920.

XIII. Sugars and Candies—Miller, Bergeim, Rehfuss, Hawk: *Am. J. Physiol.*, 53, 65 1920.

XIV. The Response of the Normal Human Stomach to Various Standard Foods and a Summary. Hawk, Rehfuss and Bergeim: *Amer. Jour. Med. Sci.*, 171, 359, 1926.

[1] Unpublished data.

data relative to the *evacuation time* and the *highest total acidity* after the ingestion of certain standard foods by normal men. In the tests here summarized 100-gram portions of food (unless otherwise stated) were fed to normal men and the gastric response determined by the fractional method (see p. 224).

HIGHEST TOTAL ACIDITIES FOR VARIOUS ARTICLES OF DIET

ARTICLES OF DIET 100-gram portions unless otherwise stated	NUMBER OF OBSERVATIONS	HIGHEST TOTAL ACIDITY (average) (c.c. N/10 alkali to neutralize 100 c.c. juice)
Beef and beef products	25	120.0
Bread and cereals	75	80.0
Cakes	29	90 0
Chicken[1]	20	125.0
Egg and egg combinations	90	80 0
Fish[1]	75	130.0
Fruits[1]	68	90.0
Gelatin[1] (fruit juice preparations)	5	70.0
Guinea hen[1]	2	110.0
Ice cream[1]	7	105.0
Ices[1]	4	65.0
Junket	4	65.0
Lamb and lamb products	14	135.0
Licorice	1	65.0
Milk:[1]		
Cows':		
400 c.c.	50	100.0
75 c.c.	3	45.0
Mothers':		
150 c.c.	5	60.0
225 c.c.	2	90.0
Nuts[1] (25 to 50 gm.)	22	100.0
Orange-albumin (2:1)	2	85.0
Pies	29	90.0
Pop corn	3	60.0
Pork and pork products	31	120.0
Puddings	23	90.0
Sugars and candies	28	70.0
Turkey[1]	2	140.0
Veal:[1]		
(*a*) Market	7	140.0
(*b*) "Bob"	7	110.0
Vegetables prepared in different ways	124	75.0

The relationship of the average evacuation times of the various foods is shown graphically in Fig. 55 whereas the variation in the evacu-

[1] Unpublished data.

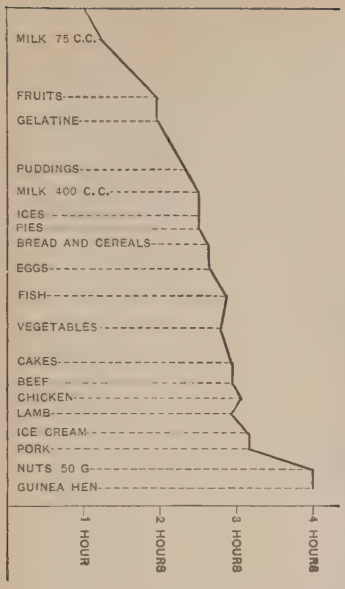


FIG. 55.—RELATIONSHIPS OF THE NORMAL EVACUATION TIMES OF STANDARD FOODS.[1]

(From Hawk, Rehfuss and Bergeim: *Am. Jour. Med. Sci.*, 171, 359, 1926.)

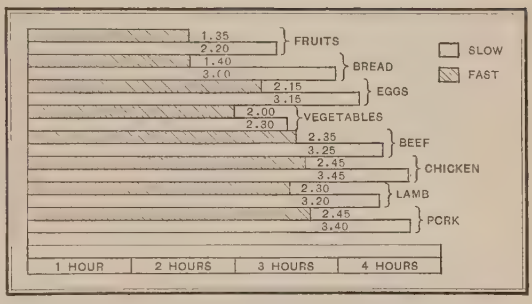


FIG. 56.—THE EVACUATION TIMES OF FAST AND SLOW STOMACHS.

(From Hawk, Rehfuss and Bergeim: *Am. Jour. Med. Sci.*, 171, 359, 1926.)

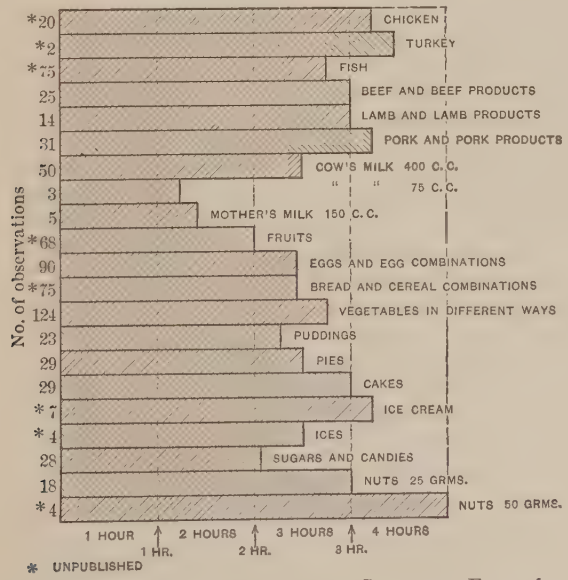


FIG. 57.—EVACUATION TIMES OF STANDARD FOODS.[1]

(From Hawk, Rehfuss and Bergeim: *Am. Jour. Med. Sci.*, 171, 359, 1926.)

[1] The average evacuation time of vegetables should be 2¼ hours instead of 2¾ hours as given in Figures 55 and 57.

ation time of the same food by *fast* and *slow* stomachs is shown in Fig. 56. Another graphic representation of evacuation times will be found in Fig. 57.

COLLECTION OF HUMAN GASTRIC JUICE

Have one or more volunteers from the class take the Rehfuss Stomach Tube as directed on page 225. The subjects must omit breakfast if the tube is taken in the morning or luncheon if taken in the afternoon. Empty the stomach (see pp. 226–229) and, with the tube still in place, allow each subject to drink 250 c.c. of water. The water will stimulate the flow of gastric juice and will itself quickly leave the stomach. In many instances fairly concentrated gastric

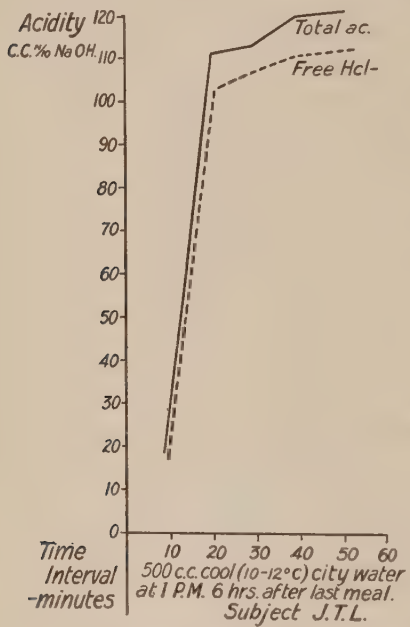

Acidity
C.C.% NaOH
Total ac.
Free Hcl
Time Interval -minutes
500 c.c. cool (10-12°C) city water at 1 P.M. 6 hrs. after last meal. Subject J.T.L.

FIG. 58.—CURVES SHOWING STIMULATORY POWER OF WATER.

(Bergeim, Rehfuss and Hawk: *Journal Biological Chemistry*, 19, 345, 1914.)

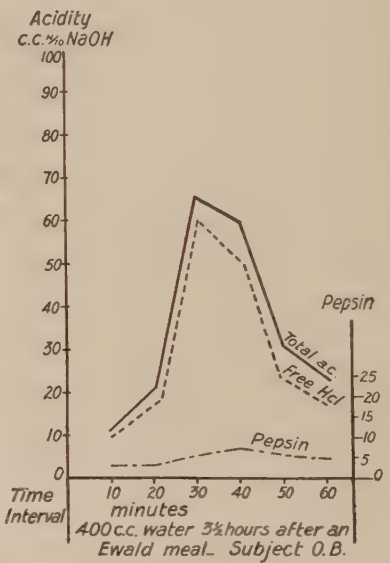


FIG. 59.—CURVES SHOWING STIMULATORY POWER OF WATER.

(Bergeim, Rehfuss and Hawk: *Journal Biological Chemistry*, 19, 345, 1914.)

juice may be obtained from the stomach in from 30 to 45 minutes after the introduction of the water. Remove this gastric juice according to procedure outlined on pp. 226–229. For composition of human gastric juice see page 221. See also Experiment 11, page 214. If thought desirable the gastric juice resulting from psychical stimulation (see page 216) may be collected instead of that following the chemical stimulation of water. (Curves showing the stimulatory power of water are given in Figs. 58 and 59.)

PREPARATION OF AN ARTIFICIAL GASTRIC JUICE

Dissect the mucous membrane of a pig's stomach from the muscular portion and discard the latter. Divide the mucous membrane into two parts (4/5 and 1/5). Cut up the larger portion, place it in a large-sized beaker with 0.4

per cent hydrochloric acid and keep at 38°–40°C. for at least 24 hours. Filter off the residue, consisting of nuclein and other substances, and use the filtrate as an artificial gastric juice. This filtrate contains pepsin, rennin, and the products of the digestion of the stomach tissues, i.e., acid metaprotein (acid albuminate), proteoses, peptones, etc.

Preparation of a Glycerol Extract of Pig's Stomach

Take the one-fifth portion of the mucuos membrane of the pig's stomach not used in the preparation of the artificial gastric juice, cut it up finely, place it in a small-sized beaker and cover the membrane with glycerol. Stir frequently and allow to stand at room temperature for at least 24 hours. The glycerol will extract the pepsinogen. Separate, with a pipette or by other means, the glycerol from the pieces of mucous membrane and use the glycerol extract as required in the later experiments.

Products of Gastric Digestion

Into the artificial gastric juice, prepared as above described, place the protein material (fibrin, coagulated egg-white, or lean beef) provided for you by the instructor, add 0.4 per cent hydrochloric acid as suggested by the instructor and keep the digestion mixture at 40°C. for two to three days. Stir frequently and keep free hydrochloric acid present in the solution (for tests for free hydrochloric acid see page 224).

The original protein has been digested and the solution now contains the products of peptic proteolysis, i.e., acid metaprotein (acid albuminate), proteoses, peptones, etc. The insoluble residue may include nuclein and other substances. Filter the digestion mixture and after testing for free hydrochloric acid neutralize the filtrate with potassium hydroxide solution. If any of the acid metaprotein (acid albuminate) is still untransformed into proteoses it will precipitate upon neutralization. If any precipitate forms heat the mixture to boiling, and filter. If no precipitate forms proceed without filtering.

We now have a solution containing a mixture consisting principally of proteoses and peptones. Separate and identify the proteoses and peptones according to the directions given on page 184.

GENERAL EXPERIMENTS ON GASTRIC DIGESTION

1. Conditions Essential for the Action of Pepsin.—Prepare four test-tubes as follows:

(a) Five c.c. of pepsin solution.

(b) Five c.c. of 0.4 per cent hydrochloric acid.

(c) Five c.c. of pepsin-hydrochloric acid solution.

(d) Two or 3 c.c. of pepsin solution and 2–3 c.c. of 0.5 per cent sodium carbonate solution.

Introduce into each tube a small piece of fibrin and place them in the incubator or water-bath at 40°C. for one-half hour, carefully noting any changes which

occur.[1] (Carmine-fibrin may be used to advantage in this and the following tests under Gastric Digestion. In this case, however, the experiments should be conducted at room temperature. For directions as to the preparation of carmine-fibrin see Chapter II.) Now combine the contents of tubes (a) and (b) and see if any further change occurs after standing at 40°C. for 15–20 minutes. Explain the results obtained from these five experiments.

2. Influence of Different Temperatures.—In each of four test-tubes place 5 c.c. of pepsin-hydrochloric acid solution. Immerse one tube in cold water from the faucet, keep a second tube at room temperature and place a third in the incubator or water-bath at 40°C. Boil the contents of the fourth tube for a few moments, then cool and also keep it at 40°C. Into each tube introduce a small piece of fibrin and note the progress of digestion. In which tube does the most rapid digestion occur? Explain this.

3. The Most Favorable Acidity.—Prepare three tubes as follows:

(a) Five c.c. of 0.2 per cent pepsin-hydrochloric acid solution.

(b) Two or 3 c.c. of 0.2 per cent hydrochloric acid + 1 c.c. of concentrated hydrochloric acid + 5 c.c. pepsin solution.

(c) One c.c. of 0.2 per cent pepsin-hydrochloric acid solution + 5 c.c. of water.

Introduce a small piece of fibrin into each tube, keep them at 40°C., and note the progress of digestion. In which degree of acidity does the fibrin digest the most rapidly?

4. Differentiation between Pepsin and Pepsinogen.—Prepare five tubes as follows:

(a) Few drops of glycerol extract of pepsinogen + 2–3 c.c. of water.

(b) Few drops of glycerol extract of pepsinogen + 5 c.c. of 0.2 per cent hydrochloric acid.

(c) Few drops of glycerol extract of pepsinogen + 5 c.c. of 0.5 per cent sodium carbonate.

(d) Two or 3 c.c. of pepsin solution + 2–3 c.c. of 1 per cent sodium carbonate.

(e) Few drops of glycerol extract of pepsinogen + 5 c.c. of 1 per cent sodium carbonate.

Add a small piece of fibrin to the contents of each tube, keep the five tubes at 40°C. for one-half hour and observe any changes which may have occurred. To (a) add an equal volume of 0.4 per cent hydrochloric acid, neutralize (c), (d) and (e) with hydrochloric acid and add an equal volume of 0.4 per cent hydrochloric acid. Place these tubes at 40°C. again and note any further changes which may occur. What contrast do we find in the results from the last three tubes? On the basis of these tests what is the relative resistance of pepsin and pepsinogen to alkalis?

5. Comparative Digestive Power of Pepsin with Different Acids.—Prepare a series of tubes each containing a N/10 solution of one of the following acids: hydrochloric, sulphuric, nitric, combined hydrochloric, acetic, lactic and oxalic. To each acid add a few drops of the glycerol extract of pig's stomach and a small

[1] Digestion of fibrin in a pepsin-hydrochloric acid solution is indicated first by a *swelling* of the protein due to the action of the acid, and later by a *disintegration* and *dissolving* of the fibrin due to the action of the pepsin-hydrochloric acid. If uncertain at any time whether digestion has taken place, the solution under examination may be filtered and the biuret test applied to the filtrate. A positive reaction will signify the presence of acid metaprotein (acid albuminate), proteoses (albumoses), or peptones, the presence of any one of which would indicate that digestion has taken place.

piece of fibrin. Shake well, place at 40°C., and note the progress of digestion. In which tubes does the most rapid digestion occur?

6. Influence of Metallic Salts, etc.—Prepare a series of tubes and into each tube introduce 4 c.c. of pepsin-hydrochloric acid solution and 0.5 c.c. of one of the chemicals listed in Experiment 18 under Salivary Digestion, page 111. Introduce a small piece of fibrin into each of the tubes and keep them at 40°C. for one-half hour. Note the variations in the progress of digestion. Where has the least rapid digestion occurred?

7. Sahli's Desmoid Reaction.—This is a method for testing gastric function without using the stomach tube. The underlying principle of the test is the fact that raw catgut may be digested in gastric juice but is entirely indigestible in pancreatic juice. The test is made as follows: A methylene-blue pill is introduced into a small rubber bag and the mouth of the bag subsequently tied with catgut.[1] The small bag is then ingested immediately after the mid-day meal and the urine examined 5, 7, 9 and 18–20 hours later for methylene blue. If methylene blue is present in appreciable quantity, it will impart to the urine a greenish-blue color. If not present in sufficient amount to impart this color the urine should be boiled with one-fifth its volume of glacial acetic acid, whereupon a greenish-blue color results if the chromogen of methylene blue is present. This contingency seldom arises, however, inasmuch as in most cases of uncolored urine it will be found that the rubber bag has passed through the stomach unopened. If the methylene blue is found in the urine inside of 18–20 hours a satisfactory gastric function is indicated.

For Einhorn's bead method for the study of digestive function see chapter on Feces.

8. Testing the Motor and Functional Activities of the Stomach.—This test is performed the same as Experiment 19 under Salivary Digestion, page 111. If the experiment was carried out under salivary digestion it will not be necessary to repeat it here.

This test does *not* serve as an index of gastric *absorption* as is sometimes stated It has been shown by von Mering, for example, that potassium iodide is not absorbed from the stomach for 2–3 hours. Hence when iodine appears in the saliva a few minutes after the KI is ingested this fact would indicate absorption *from the intestine*. The gastric mucosa is not importantly related to absorption.

9. Influence of Bile.—Prepare three tubes as follows:

(a) Five c.c. of pepsin-hydrochloric acid solution + 0.5–1 c.c. of bile.

(b) Five c.c. of pepsin-hydrochloric acid solution + 5 c.c. of bile.

(c) Five c.c. of pepsin-hydrochloric acid solution.

Introduce into each tube a small piece of fibrin. Keep the tubes at 40°C. and note the progress of digestion. Does the bile exert any appreciable influence? How?

[1] About 0.05 gram of methylene blue is mixed with sufficient *ext. glycyrrhizæ* to form a pill about 3–4 mm. in diameter. The pill is then placed in the center of a square piece of thin rubber dam and a little bag-like receptacle constructed by a twisting movement. The neck of the bag is then closed by wrapping three turns of catgut about it. The most satisfactory catgut to use is *number* 00 *raw catgut* which has previously been soaked in water until soft. When ready for use the bag should sink instantly when placed in water and be water-tight.

10. Influence of Gastric Rennin on Milk.—Prepare a series of five tubes as follows:

(a) Five c.c. of fresh milk + 0.2 per cent hydrochloric acid (add slowly until precipitate forms).

(b) Five c.c. of fresh milk + 5 drops of rennin solution.[1]

(c) Five c.c. of fresh milk + 10 drops of 0.5 per cent sodium carbonate solution.

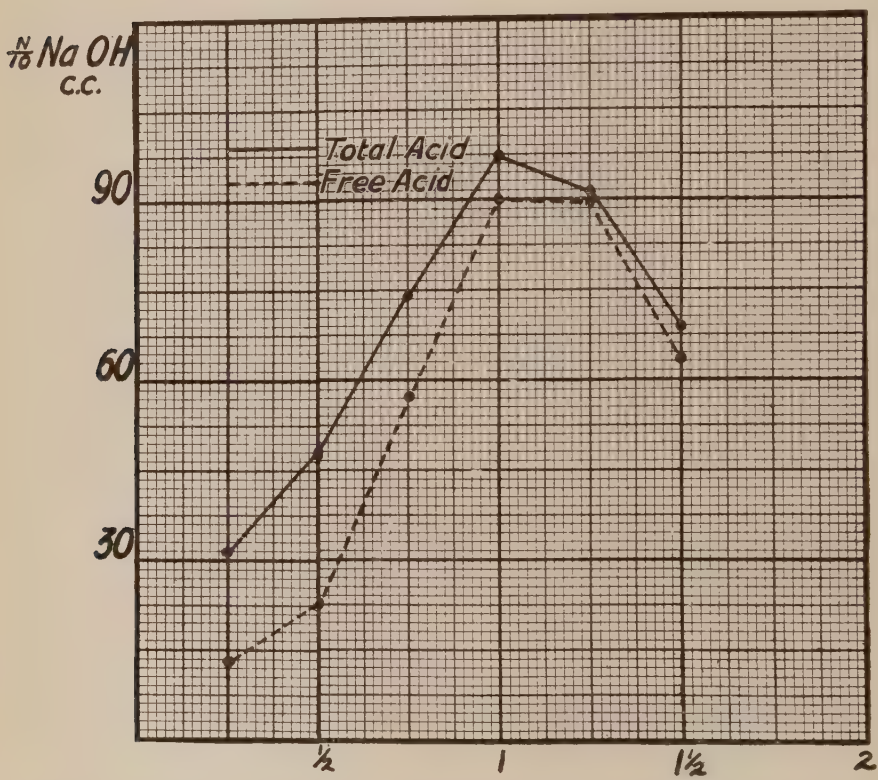


FIG. 60.—CURVES SHOWING STIMULATORY POWER OF BEEF EXTRACT.
(From unpublished data collected in the senior author's laboratory by Dr. Chester C. Fowler.)

(d) Five c.c. of fresh milk + 10 drops of a saturated solution of ammonium oxalate.

(e) Five c.c. of fresh milk + 5 drops of 0.2 per cent hydrochloric acid. Now to each of the tubes (c), (d), and (e) add 5 drops of rennin solution. Place the whole series of five tubes at 40°C. and after 10–15 minutes note what is occurring in the different tubes. Give a reason for each particular result. How do ammonium oxalate and sodium carbonate prevent coagulation?

11. Characteristics of Human Gastric Juice.—Take some of the human gastric juice collected as described on page 210 and show that it is acid in

[1] Any good commercial rennin or rennet preparation may be used in preparing this solution.

reaction, that it contains chlorides and that it has the power to digest protein material and to curdle milk.

12. Chemical Stimulation of Gastric Secretion.—Have one or more volunteers from the class take the Rehfuss Stomach Tube as directed on page 225. The subjects must omit breakfast if the tube is taken in the morning or luncheon if taken in the afternoon. Empty the stomach (see pp. 226–229) and, with the

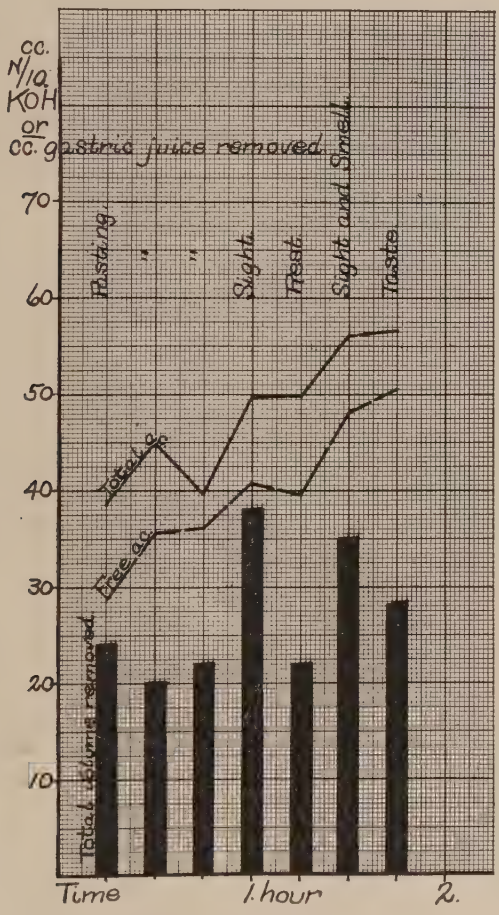


Fig. 61.—Curves Showing Psychical Stimulation of Gastric Secretion. (*From unpublished data collected in the senior author's laboratory by Dr. Raymond J. Miller.*)

tube still in position, allow each subject to drink 250 c.c. of bouillon prepared by dissolving one bouillon cube in hot water. Collect samples of gastric contents at intervals until the stomach is empty as described under 5 on page 229. The samples thus collected may be examined qualitatively for acid, chlorides, pepsin and rennin or they may be submitted to the quantitative procedure given under 6 on page 229. If the examination is made quantitative the data may be recorded in the form of a curve such as shown in Fig. 60, page 214.

13. Psychical Stimulation of Gastric Secretion.—Have one or more volunteers from the class take the Rehfuss Stomach Tube as directed on page 225. The subjects must omit breakfast if the tube is taken in the morning or luncheon if taken in the afternoon. Empty the stomach (see pp. 226–229) and while the tube is still in position permit the subjects to see and smell an appetizing beefsteak while it is being cooked. They may also be permitted to taste some of the material provided care is taken that none is swallowed. Empty the stomach completely at 10 minute intervals as described under 5 on page 229. Measure the volume of each sample and examine them qualitatively for acid, chlorides, pepsin and rennin. If preferred the quantitative procedure given under 6 on page 229 may be substituted for the qualitative examination. On the basis of the quantitative data a curve such as shown in Fig. 61 may be constructed.

14. Automatic Regulation of Gastric Acidity.—Have one or more volunteers from the class take the Rehfuss Stomach Tube as directed on page 225. The subjects must omit breakfast if the tube is taken in the morning or luncheon if taken in the afternoon. Empty the stomach (see pp. 226–229) and, with the tube still in position, introduce 100 c.c. of 0.5 per cent HCl through the tube by means of a syringe. Withdraw samples of stomach contents at 15 minute intervals, until the stomach is empty, as described on page 229. A qualitative examination of the samples will indicate a progressive decrease in acidity until an acid concentration in the neighborhood of 0.2–0.3 per cent is reached. Trypsin (p. 237) and bile (p. 240) may also be shown to be present, in at least some of the samples, thus indicating regurgitation from the duodenum. If it is desired to make the examination quantitative the procedure described on page 229, section 6, may be followed. From the data thus determined a curve such as that shown in Fig. 69, page 230, may be constructed. (For a discussion of regurgitation see pages 220 and 237.)

CHAPTER IX

GASTRIC ANALYSIS

The method of gastric analysis which was in vogue clinically for years (see page 242) entailed the feeding of a standard test meal, the removal of the complete stomach contents at the end of a *one-hour period*, and the analysis of the material so removed. That this method is inaccurate has been repeatedly demonstrated in the author's laboratory[1] and elsewhere.[2] Furthermore, owing to the bulk of the old form of stomach tube and the discomfort occasioned by its use, it is impossi-

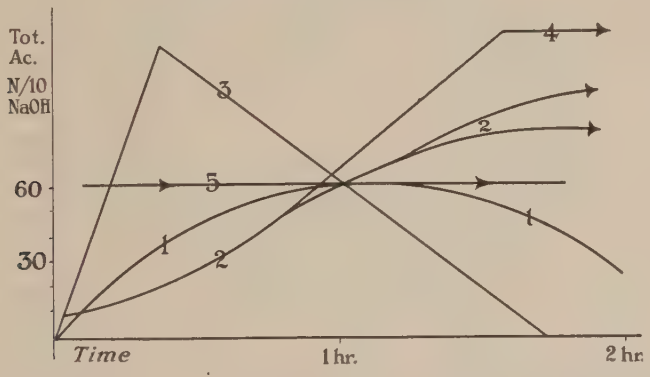


Fig. 62.—Normal and Pathological Curves after an Ewald Meal.
1, normal curve; 2, delayed digestion with late hyperacidity; 3, larval hyperacidity; 4, tardive hyperacidity; 5, marked continued secretion from obstruction.

ble to follow the *whole cycle* of digestion and estimate, step by step, the exact changes which take place in the stomach after the introduction of definite food mixtures into that organ.

Realizing the inadequacy of the procedure entailed in the old method of gastric analysis, a new procedure was developed by Dr. Martin E. Rehfuss in the senior authors' laboratory. This so-called "Fractional Method" entails the analysis of samples of material withdrawn from the stomach (by syringe) at short intervals for a period of two hours or more (until stomach is empty) after the ingestion of the test meal. By this means the observer is able to follow the *entire cycle* of

[1] Rehfuss: *Jour. Am. Med. Ass'n*, 64, 569, 1914.
Rehfuss, Bergeim and Hawk: *Jour. Am. Med. Ass'n*, 63, 909, 1914.
Bergeim, Rehfuss and Hawk: *Jour. Am. Med. Ass'n*, 63, 11, 1914.
[2] Harmer and Dodd: *Arch. Int. Med.*, Nov. 13, 1913, p. 488.

gastric digestion and is not limited, as in the old method, to information derived from the analysis of a single sample of stomach contents withdrawn at the end of one hour. That the acid values obtained by the old method may be grossly misinterpreted and lead to an incorrect diagnosis is indicated by the foregoing diagram (Fig. 62).

It is set forth in the above diagram that various types of abnormal gastric secretion would be considered normal on the basis of a single examination at the end of one hour whereas the application of the *fractional method* reveals the abnormality of the secretion and enables a rapid and correct diagnosis. The removal of samples of gastric contents at short intervals, for a period of two hours or more after a test meal, is made possible by the use of a modified stomach tube[1] of small diameter (No. 12 French tubing) and fitted with a metal tip.

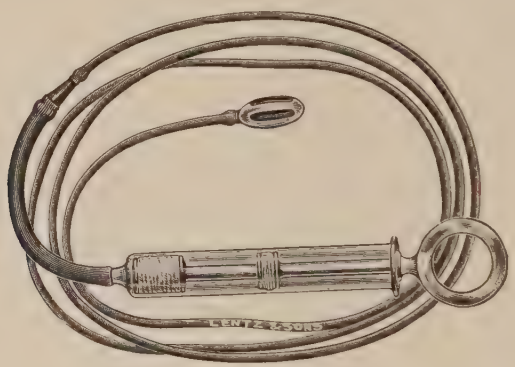


Fig. 63.—Rehfuss Stomach Tube.

The tip is slotted with large perforations, the diameter of each being equivalent to the maximum bore of the tubing. Such a tube can be left in the stomach through the entire cycle of gastric digestion without inconvenience to the patient.[2] A cut of the Rehfuss stomach tube (Fig. 63) is shown above.[3] Lyon has suggested a modified tip.[4]

A tube much favored in England is that devised by Ryle.[5] This consists of a small bore rubber tube with a blind end, into which is inserted an oval weight of lead (See Fig. 64). Holes are punched in the rubber tube just above the weight. Ryle claims the following advantages over a metal-tipped tube: (1) more easily swallowed and withdrawn; (2) blockage with mucus plugs generally avoided; (3) impos-

[1] Rehfuss: *Am. Jour. Med. Sci.*, June, 1914.

[2] McClendon has suggested the introduction of an electrode into the stomach in an attempt to follow the consecutive changes in the hydrogen ion concentration of the stomach contents (see *Am. Jour. Physiol.*, 38, 180, 1915).

[3] This tube is manufactured by Charles Lentz & Sons, Philadelphia.

[4] Lyon: *J. Am. Med. Ass'n.*, 74, 246, 1920.

[5] Ryle: Gastric Function in Health and Disease, Oxford University Press, London, 1926.

sible to detach end; (4) less likelihood of damaging the gastric mucosa; (5) cheaper. This tube is manufactured by Down Bros., 21 St. Thomas St., S. E., London, England. For further discussion of the Fractional Method see p. 224.

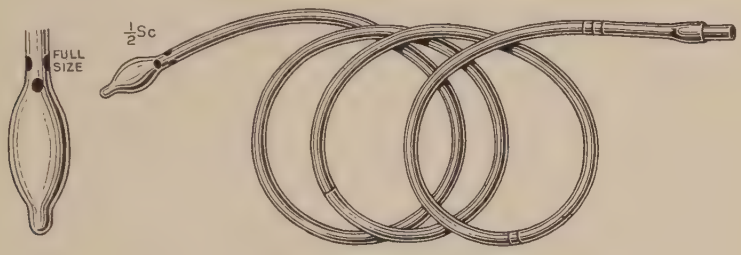


FIG. 64.—RYLE'S GASTRIC TUBE.
(From Ryle "Gastric Function in Health and Disease," Oxford University Press, London, 1926.)

An apparatus has also been devised[6] for the determination of *intragastric conductance* and *temperature*. The apparatus is also provided with an aspiration tube similar to that of the Rehfuss tube which makes

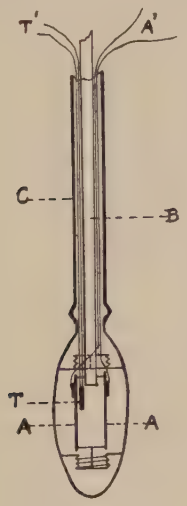


FIG. 65.

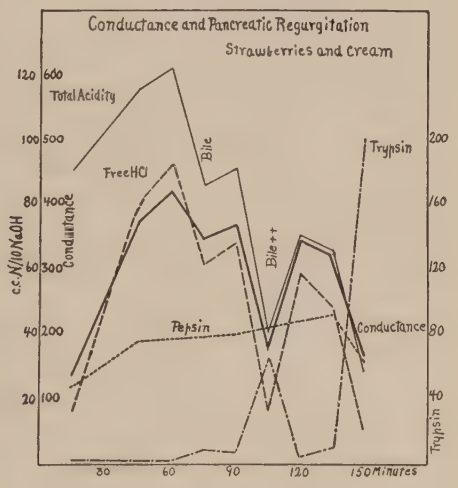


FIG. 66.

FIG. 65.—BERGEIM INTRAGASTRIC CONDUCTANCE APPARATUS.
Diagrammatic cross-section showing platinum electrodes *A*, *A*, with leads *A'*; thermocouple *T* with leads, *T'*; tube for aspiration *B*; and outer protecting tube *C*. (*Bergeim: American Journal of Physiology*, 45, 1, 1917.)

FIG. 66.—CURVES SHOWING RELATIONSHIP OF CONDUCTANCE TO ACIDITIES.
(*Bergeim: American Journal of Physiology*, 45, 1, 1917.)

possible the removal of samples of gastric contents for chemical analysis. This apparatus is shown in Fig. 65, above. Curves showing the relationship of conductance to acidities are given in Fig. 66, above.

[6] Bergeim: *Amer. Jour. Physiol.*, 45, 1, 1917

For a long time, the consensus of opinion based principally upon the work of the Pawlow school[1] was to the effect that the gastric juice of normal man had an average acid concentration of 0.2 per cent hydrochloric acid, whereas the gastric juice of the dog and cat had an average acid concentration of 0.4–0.5 per cent hydrochloric acid. These experiments were based principally upon the examination of the pure gastric juice of the lower animals as compared with the stomach contents of man. Later experiments,[2] however, demonstrated that the acid concentration of the *freshly secreted* gastric juice of man is similar to that of the dog, *i.e.*, 0.4–0.5 per cent. Boldyreff claimed that this

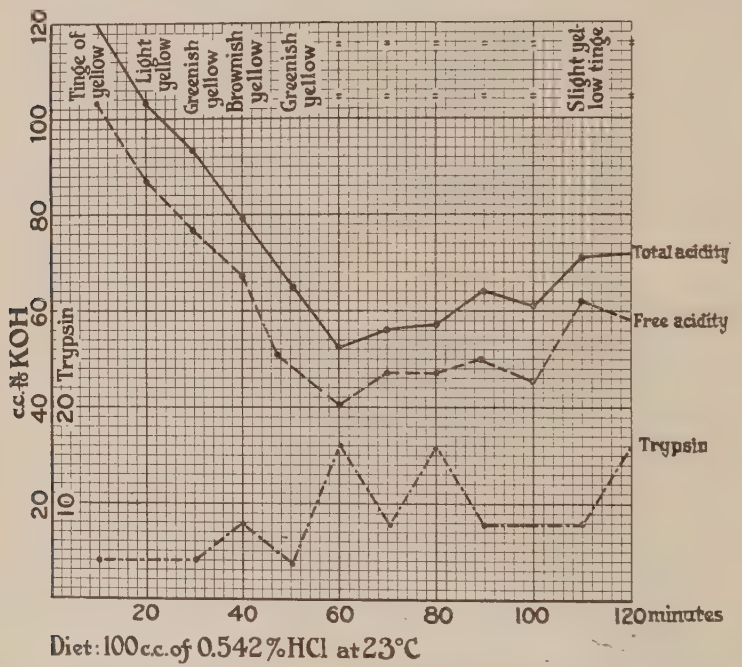


FIG. 67.—INFLUENCE OF ACID INTRODUCED INTO THE NORMAL HUMAN STOMACH. (*Spencer, Meyer, Rehfuss and Hawk: American Journal of Physiology*, 39, 459, 1916.)

initial high acidity of the human gastric juice is normally lowered to the "optimum acidity" of 0.15–0.2 per cent hydrochloric acid by *regurgitation of alkaline fluids* (bile, pancreatic and intestinal juices) from the intestine. This constitutes what Boldyreff termed "*the automatic regulation of gastric acidity.*" This claim has been substantiated by

[1] Pawlow: *The Work of the Digestive Glands.* Translated by Thompson, Second Edition 1910.

[2] Babkin: *Die Äussere Sekretion der Verdauungsdrüsen*, Berlin, 1914.
Boldyreff: Transactions of 11th Congress of Physicians, St. Petersburg, 1909.
Boldyreff: *Quart. Jour. Exp. Physiol.*, 8, 1, 1914.
Carlson: *Am. Jour. Physiol.*, 38, 248, 1915.
Bergeim, Rehfuss & Hawk: *Jour. Biol. Chem.*, 19, 345, 1914.

experiments made in the senior author's laboratory[1] and elsewhere.[2] Both bile and trypsin are easily identified in the stomach contents of man after the introduction of 0.5 per cent hydrochloric acid into the empty organ. The above points are illustrated by the chart shown in Fig. 67, page 220.[3]

The composition of human gastric juice and of the residuum (see page 226) is given in the following table:

COMPOSITION OF HUMAN GASTRIC JUICE

Constituent	Appetite juice[4]	Residuum[5]
Specific gravity	1.007	1.006
Δ degrees	−0.55	−0.47
Total acidity, per cent	0.45	0.30
Per 100 c.c. of Juice		
Total solids, gram	0.55	0.98[6]
Organic solids, gram	0.41	0.53[6]
Inorganic solids, gram	0.14	0.45[6]
Total nitrogen, gram	0.060	0.066[6]
Total phosphorus, gram		0.005[6]
Total sulphur, gram		0.007[6]
Ammonia N, gram	0.002–3	
Amino-acid N, gram	0.003–9	
Chlorides (as Cl), gram	0.5	

The Use of Indicators in Determining the Reaction of Gastric Juice and Other Fluids[7]

Indicators behave like very weak acids, whose salts are of different colors from those of the acids. They exhibit buffer properties over a range of hydrogen ion concentration characteristic for each indicator. As the proportions of acid and salt forms of indicator change over this range, a graded scale of colors results, starting with the color of the pure acid form and ending with the color of the pure salt (alkaline) form. The pH limits between which this change occurs determine which indi-

[1] Spencer, Meyer, Rehfuss and Hawk: *Am. Jour. Physiol.*, 39, 459, 1916.
[2] Migai: *Diss.*, St. Petersburg, 1909.
Milosorov: *Zent. Physiol.*, 28, 615, 1914.
Zaitzeff: *Russky Vrach.*, 14, No. 29, 1915.
[3] Spencer et al: *Loc. cit.*
[4] Carlson: *Loc. cit.*
[5] Fowler, Rehfuss and Hawk: *Loc. cit.*
[6] Fowler and Buchanan: Unpublished.
[7] For a general discussion of indicators, hydrogen ion concentration and titratable acidity, see Chapter I.

cator may be selected for a particular titration. Indicators which change color at the approximate true neutral point are litmus and bromthymol blue, while phenolphthalein and phenolsulphonephthalein change color in slightly alkaline solution. Methyl red, sodium alizarin sulphonate and bromcresol green are examples of indicators which change color in an acid solution.

The salts of weak *organic acids* hydrolyze in dilute solution so that the correct end-point in the titration of such an acid with a strong base is on the alkaline side of the neutral point. Hence it is necessary to use an indicator like phenolphthalein which changes in slightly alkaline solution, and not one like methyl red or alizarin, whose color change is already passed by the time the actual neutral point is reached. In the titration of solutions of strong *mineral acids*, whose hydrogen ion concentration in dilute solution is much greater, a wide range of pH is covered, making it possible to use a greater selection of indicators. Methyl red, bromcresol green or dimethylaminoazobenzene (Töpfer's reagent) change color on the acid side of neutrality and are therefore useful in titrating a mixture of a mineral acid and an organic acid up to the point of neutralization of the former alone. By a judicious selection of indicators it is thus possible to titrate a mixture of acids, such as is contained in gastric juice, and obtain the total acidity, free mineral acid, organic acid, combined acidity, etc.

Indicator	pH range	Color change
Thymol blue (acid range)	1.2– 2.8	Red-yellow
Töpfer's reagent	2.9– 4.0	Red-yellow
Bromphenol blue	3.0– 4.6	Yellow-blue
Congo red	3.0 –5.0	Blue-red
Methyl orange	3.1– 4.4	Orange red-yellow
Bromcresol green	4.0– 5.6	Yellow-blue
Methyl red	4.2– 6.3	Red-yellow
Litmus	4.5– 8.3	Red-blue
Chlorphenol red	5.0– 6.6	Yellow-red
Alizarin red	5.0– 6.8	Yellow-red
p-Nitrophenol	5.0– 7.0	Colorless-yellow
Bromcresol purple	5.4– 7.0	Yellow-purple
Bromthymol blue	6.0– 7.6	Yellow-blue
Phenol red	6.6– 8.2	Yellow-red
Neutral red	6.8– 8.0	Red-yellow
Cresol red	7.2– 8.8	Yellow-red
Metacresol purple	7.6– 9.2	Yellow-purple
Thymol blue (alkaline range)	8.2– 9.8	Yellow-blue
Phenolphthalein	8.3–10.0	Colorless-red
Alizarin yellow	10.0–12.0	Colorless-yellow
Tropaeolin O	11.1–12.7	Yellow-orange

TABULATION OF RESULTS OF TESTS ON INDICATORS[1]

Solution	Approximate normality	Approximate pH	1 Thymol blue	2 Methyl orange	3 Töpfer's reagent	4 Sahli's reagent ($KI-KIO_3$) (2 c.c.)	5 Congo red	6 Alizarin	7 Litmus	8 Neutral red	9 Phenol red	10 Cresol red	11 Phenol-phthalein	12 Günzberg's reagent
1. 0.4 per cent HCl.....	N/ 10	1.0												
2. 0.04 per cent HCl.....	N/100	2.0												
3. 0.6 per cent acetic acid.....	N/ 10	3.0												
4. 0.04 per cent combined HCl[2].	N/100	4.0												
5. Acid phosphate 1:9[3].....	M/ 15	6.0												
6. Water.....	.....	7.0												
7. Basic phosphate 20:1[3].....	M/ 15	8.0												
8. Borate-NaOH 6:4[4].....	N/ 10	10.0												
9. NaOH 0.4 per cent.....	N/ 10	13.0												

[1] Indicator solutions. *Thymol blue, Phenol red and Cresol red* 0.04 gram in 100 c.c. water. *Methyl orange*, 0.1 gram in 100 c.c. water. *Töpfer's reagent*, 0.5 gram dimethyl-amino-azo-benzene in 100 c.c. 95 per cent alcohol. $KI-KIO_3$, mix equal volumes of 8 per cent KIO_3 and 48 per cent KI solutions. *Congo red*, 0.5 gram in 90 c.c. water and add 10 c.c. 95 per cent alcohol. *Alizarin*, 1 gram sodium alizarin sulphonate in 100 c.c. water. *Litmus*, preferably azo-litmin 1 per cent solution in water. *Neutral red*, 0.05 gram in 50 c.c. 95 per cent alcohol, add 50 c.c. water. *Phenolphthalein*, 1 gram in 100 c.c. 95 per cent alcohol.

[2] Combined HCl. Treat 0.04 per cent HCl with a small amount of Witte's peptone and boil until the solution no longer gives a blue but only a brown color with Congo red paper.

[3] Make up a solution of potassium dihydrogen phosphate of $\frac{1}{15}$ molecular strength (9.078 grams to a liter of water) and one of disodium hydrogen phosphate (obtained by drying the ordinary salt in the air for a few weeks) $Na_2HPO_4.2H_2O$ of similar strength (11.876 grams to a liter). To prepare the acid phosphate solution used in the test mix 1 part of the solution of the disodium salt with 9 parts of the solution of the dihydrogen phosphate. For the basic phosphate solution the proportions are 20:1.

[4] Borate-NaOH solution. Prepare a borate solution by dissolving 12.404 grams of pure boric acid (0.2 mol.) in 100 c.c. N NaOH solution and dilute with water to a liter. Prepare the Borate-NaOH solution by mixing 6 parts of the borate solution with 4 parts of N/10 NaOH.

Tests with Indicators.—Prepare a series of solutions of varying acidities as outlined in the preceding table, page 223. Introduce 5 or 10 c.c. portions of each of these into a series of test-tubes and add to each a few drops of a solution of thymol blue. Make a note of the colors produced, in the spaces left for this purpose. In the same way test out the other indicators mentioned, in order, using in each case a few drops of the indicator solution.

Are the following assumptions, on which the use of certain of these indicators in gastric analysis is based, borne out by your findings?

1. That Töpfer's reagent (dimethylaminoazobenzene) gives its characteristic pinkish-red color only in the presence of free HCl.

2. That a blue color with Congo red indicates free hydrochloric (or other mineral acid), a violet color indicates an organic acid, and a brown color indicates combined hydrochloric acid.

3. That thymol blue and methyl orange are indicators for free mineral acid.

4. That alizarin reacts to free mineral acid, organic acids and acid salts but not to combined HCl.

5. That phenolphthalein can be used in titrating total acidity, that is, acidity due to mineral and organic acids, acid salts and combined acid.

6. That iodine is liberated from $KI–KIO_3$ to a relatively slight extent by other than free mineral acid.

7. That Günzberg's test is the most satisfactory one for free HCl.

Special Tests for Free HCl.—Perform the following tests on the solutions as outlined above and tabulate the results.

1. Günzberg's Reagent.[1]—Place 1–2 drops of the reagent in a small porcelain evaporating dish and carefully evaporate to dryness over a low flame. Insert a glass stirring rod into the mixture to be tested and draw the moist end of the rod through the dried reagent. Warm again gently and note the production of a purplish-red color in the presence of free hydrochloric acid.

THE FRACTIONAL METHOD OF GASTRIC ANALYSIS

Since 1914, when the first experiments entailing the use of the fractional method were reported from the senior author's laboratory, the method has been widely adopted both in this country and abroad. This wide-spread use by a large number of workers has resulted in much discussion of the method.[2] On the basis of this large experience certain

[1] Günzberg's reagent is prepared by dissolving 2 grams of phloroglucinol and 1 gram of vanillin in 100 c.c. of 95 per cent alcohol.

[2] Rehfuss: Diseases of the Stomach, Saunders, Philadelphia, 1926.
Ryle: Gastric Function in Health and Disease; Oxford University Press, London, 152 pp., 1926.
Ellison: *Am. Jour. Med. Sci.*, 169, 550, 1925.
Larimore: *Jour. Lab. Clin. Med.*, 11, 1, 1925.
Hubbard and Lichty: *Ann. Clin. Med.*, 4, 393, 1925.

modifications in the original technique are here presented. In its major details however, the method remains as originally carried out and constitutes, in the opinion of the authors, the best available method for the clinical examination of the stomach.

Procedure in Gastric Analysis by the Fractional Method

1. Introduction of the stomach tube (see below).
2. Removal of the residuum (see page 226).
3. Feeding the test meal (see page 228).
4. Feeding the retention meal (in special cases), see page 228.
5. Removing samples of stomach contents for analysis (see p. 229).
6. Examination of the samples for:
   - (*a*) Total acidity (see page 229).
   - (*b*) Free acidity (see page 231).
   - (*c*) Hydrogen ion concentration (see page 233).
   - (*d*) Pepsin (see page 234).
   - (*e*) Trypsin (not a routine procedure), see page 237.
   - (*f*) Lactic acid (see page 238).
   - (*g*) Occult blood (see page 239).
   - (*h*) Bile (see page 240).
   - (*i*) Microscopical constituents (see page 241).

1. Introduction of the Stomach Tube.—Whereas the large tube is directly inserted by propulsion, the Rehfuss tube is swallowed in the natural manner and aided by gravity. The tube may be passed in one of three ways, *i.e.:* (1) lubricated; (2) with aid of fluid; (3) after throat is cocainized. When passed by the first method the tip of the tube, after thorough lubrication with glycerol or liquid petrolatum, is seized between the thumb and forefinger and placed on the tongue. Then with the aid of the forefinger the tip is pushed backward until it reaches the root of the tongue and is engaged in the oropharynx. Then the patient is encouraged to breathe deeply through the nose and to swallow persistently while the tube is slowly fed into the mouth. After slight dis-

Bennett and Dodds: *Brit. Jour. Exp. Path.*, ii, 58, 1921.
Hubbard, Munford and Allen: *Am. Jour. Physiol.*, 68, 207, 1924.
Rehfuss: *Ann. Clin. Med.*, 2, 55, 1924.
Bennett and Venables: *Brit. Med. Jour.*, 2, 662, 1920.
Gorham: *Arch. Int. Med.*, 27, 434, 1921.
Wheelon: *Arch. Int. Med.*, 28, 613, 1921.
Kopeloff: *Arch. Int. Med.* 30, 118, 1922.
Kopeloff: *Jour. Am. Med. Ass'n*, 78, 404, 1922.
Jones: *Jour. Am. Med. Ass'n* (Discussion of Eggleston's paper see below).
Hurst: *Lancet*, 1, 111, 1923.
Eggleston: *Jour. Am. Med. Ass'n*, 83, 260, 1924.
Friedenwald and Bryan: *Jour. Am. Med. Ass'n*, 83, 265, 1924.
Lyon, Barth and Ellison, *New York Med. Jour.*, 114, 272, 1921.
White: *Jour. Am. Med. Ass'n*, 79, 1499, 1922.
Friedenwald, Gannt and Morrison: *Ann. Clin. Med.*, 2, 292, 1924.

comfort in the pharynx and its passage past the level of the cricoid cartilage, practically no discomfort is felt. This method is used when it is essential that the pure gastric secretion or residuum be obtained. Ordinarily, however, it is much easier to swallow the tube by the second method. This method consists in placing the tip in the oropharynx and then giving the patient a measured quantity of water or tea to swallow. The movements induced by the swallowing carry the tube rapidly to the stomach with a minimum of discomfort. When an Ewald meal (see p. 228) is given, part of the tea may be reserved for swallowing the tube. This procedure makes it scarcely more arduous then the swallowing of food. Should the patient, however, be extremely neurotic or the unfortunate possessor of marked pharyngeal hyperesthesia, cocain hydrochloride in 2 per cent aqueous solution may be applied to the throat rendering the passage of the tube practically insensitive. When the tube has entered the stomach, aspiration of the material shows the characteristic gastric contents. Should the tip remain in the esophagus through transient cardiospasm or other cause, aspiration results in the removal of only a very small specimen having all the characteristics of the pharyngeal and esophageal secretions.

The clinician should see to it that the tube is so placed that the tip reaches the lower pole of the stomach. Furthermore *the tip should remain in this position throughout the duration of the test.* By placing the tube in this manner, representative specimens and accurate data may be obtained.

That the gastric response to the fractional method when properly performed is remarkably similar for the same individual on different days is shown in Fig. 68.

Bloomfield and Keefer[1] have suggested a method for the "continuous quantitative estimation of gastric secretion and discharge in man."

2. Removal of Residuum.—If the so-called "empty" stomach is examined in the morning before any food or drink has been taken it will be found to contain considerable material. This is termed residuum. Before a test meal is introduced into the stomach, this organ should be emptied. If this is not done we cannot consider the samples withdrawn after the test meal is eaten as representing the secretory activity of the gastric cells under the influence of the stimulation of the test meal. It has been generally recognized, clinically, that a residuum above 20 c.c. is pathological.[2] Such a volume has been considered as

[1] Bloomfield and Keefer: *Arch. Int. Med.*, 37, 819, 1926.

[2] Loeper: *Leçons de pathologie digestive*, 1912, Series 2, pp. 17–19.
Zweig: Magen- und Darmkrankheiten, p. 459.
Kemp: Diseases of the Stomach, Intestines and Pancreas, 1912, p. 133.
Wolff: Taschenbuch der Magen- und Darmkrankheiten, p. 22.

indicative of hypersecretion, and this in turn in many cases indicates an organic lesion. The observations indicating that a residuum of over 20 c.c. was pathological, were made upon residuums removed by means of the old type of stomach tube which does not completely empty the stomach.[1] When the residuum is completely removed by means of the Rehfuss tube it has been demonstrated that the *normal* residuum

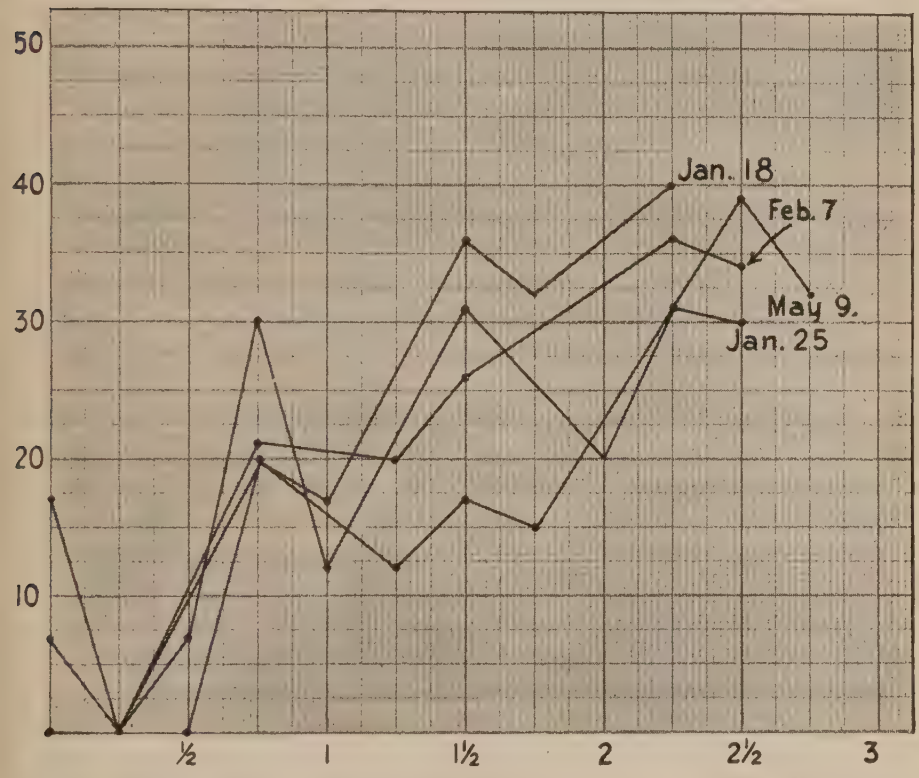


FIG. 68.—SHOWING GASTRIC RESPONSE OF SAME INDIVIDUAL ON FOUR DIFFERENT OCCASIONS.

(After Ryle, "Gastric Function in Health and Disease," Oxford University Press, London, 1926.)

is practically always over 20 c.c. and that the average is about 50 c.c. for both men[2] and women.[3] The normal residuum has been found to possess all the qualities of a physiologically active gastric juice with an average total acidity of 30 and an average free acidity of 18.5. The residuum is often colored by bile. This is particularly true if the fluid has a relatively high acidity. Trypsin is also generally present.

[1] Harmer and Dodd: *Loc. cit.*
[2] Rehfuss, Bergeim and Hawk: *Jour. Am. Med. Ass'n.*, 63, 11, 1914.
Fowler, Rehfuss and Hawk: *Jour. Am. Med. Ass'n.*, 65, 1021, 1915.
[3] Fowler and Zentmire: *Jour. Am. Med. Ass'n.*, 68. 167, 1917.

These findings indicate regurgitation (see page 220). Pathological residuums may contain blood, pus, mucus and may show food retention which is indicative of disturbed food evacuation. The quantity may also be much increased due to hypersecretion. A residuum of large volume possessing a total acidity value of 70 or over may indicate ulcer.

Analysis of Residuum.—Remove the residuum as directed under (5) p. 229, and analyze the fluid according to methods outlined on page 229 et seq.

3. The Test Meal.—Before making an analysis of the stomach contents it is customary to introduce something into the stomach which will stimulate the gastric cells. The response to this stimulation is then measured clinically by the determination of total acidity, free acidity and pepsin in the stomach contents. Many forms of test meal have been used.

The test meal most widely employed is the Ewald test meal. This consists of 2 pieces (35 grams) of toast and 8 ounces (250 c.c.) of tea.

Inasmuch as it was demonstrated in the author's laboratory[1] that water gave a similar gastric stimulation to that produced by the Ewald meal it was suggested that a simple water meal might be substituted for the Ewald meal. This water meal also has the added advantage of enabling one to determine the presence of food rests and to test more accurately for lactic acid, blood and bile. Ryle[2] prefers to use oatmeal gruel (1 pint). Doses of 1 mg. of histamin hydrochloride injected subcutaneously are effective in stimulating gastric secretion and may help to differentiate between true and "apparent" achylia gastrica.[3]

4. The Retention Meal.—In order to obtain more information regarding gastric motility than is furnished by the ordinary test meal described above the patient may be fed a so-called *retention meal*. This meal is fed in place of the regular evening meal and contains substances readily detected. In the morning before breakfast (7–8 a. m.) remove the stomach contents (residuum, see page 226) by aspiration and examine for food rests. The normal stomach should give no evidences of food retention. A satisfactory retention meal consists of 4 ounces each of boiled string beans and rice.[4] Diets containing prunes, raspberry marmalade, lycopodium powder, etc., have also been employed. In many instances an ordinary mixed diet will serve the purpose.

[1] Bergeim, Rehfuss, and Hawk: *Jour. Biol. Chem.*, 19, 345, 1914.
Rehfuss, Bergeim and Hawk: *Jour. Am. Med. Ass'n*, 63, 11, 1914.
[2] Ryle: *Loc. cit.*
[3] Gompertz and Vorhaus: *J. Lab. Clin. Med.*, 11, 14, 1925.
[4] **Myers and Fine:** *Essentials of Pathological Chemistry*, 1913.

5. Removal of Samples for Analysis.—At intervals of exactly 15 minutes from the time the test meal is eaten until the stomach is empty 5–6 c.c. samples of gastric contents are withdrawn from the stomach by means of aspiration and a few cubic centimeters of air blown down the tube in order that the tube shall be empty when the next sample is drawn. If the stomach is not empty at the end of three hours, the remaining stomach contents may be withdrawn and measured.

In order to facilitate the mixing of the stomach contents and the withdrawal of a more representative sample the stomach contents should be aspirated back and forth *four* times before taking the sample for analysis. Some clinicians advise that the stomach contents be mixed by *physical manipulation* just prior to aspiration.

In the removal of samples from the stomach, it is essential that very little traction be employed. To completely empty the stomach, aspiration is practised in four positions: (*a*) on the back; (*b*) on the stomach; (*c*) on right side, (*d*) on left side. This results in complete evacuation of the stomach. Three tests may be employed to determine whether the stomach is empty: (1) No more material can be aspirated in any position; (2) injection of air and auscultation over the stomach with a stethoscope reveals a sticky rale and not a series of gurgling rales such as is heard when there is material in the stomach; (3) lavage or irrigation through the tube which shows the absence of all food in the stomach.

6. Examination of the Samples.—The old methods of gastric analysis involved the collection (by analysis and calculation) of data regarding several types of acidity (see Töpfer's method, page 242). The modern tendency among clinicians is to lay particular emphasis upon the values for total acidity and free acidity. The determination of the peptic activity is also important as well as the demonstration of the presence or absence of occult blood, lactic acid, mucus, food rests, etc.

Procedure.—Strain each sample through a fine-mesh cheese cloth.[1] Examine the residue for mucus, blood[2] and food rests. Use the strained stomach contents for the determination of total acidity, free acidity and peptic activity by methods which follow.

(a) Determination of Total Acidity.—*Principle.*—The indicator used is phenolphthalein. Since the indicator reacts with mineral acid, organic acid, combined acid and acid salts the values obtained represent the *total* acidity of the solution.

[1] The examination for microscopical constituents (see (*h*) p. 241) should be made on the original (unstrained) gastric contents. Tests for occult blood may be made on the *sediment* if desired.

[2] The detection of blood is rather more satisfactory in the residue than in the strained fluid.

Procedure.—Measure 1 c.c. of the strained stomach contents by means of an Ostwald pipette and introduce it into a low-form 60 c.c. porcelain evaporating dish. Dilute with 15 c.c. of distilled water. Add 2 drops of a 1 per cent alcoholic solution of phenolphthalein and titrate with N/100 sodium hydroxide until a faint pink color is obtained and persists for about two minutes.[1] Take the burette reading and calculate the total acidity.

Calculation.—Note the number of cubic centimeters of N/100 NaOH required to neutralize 1 c.c. of stomach contents, and multiply it by 10 to obtain the number of cubic centimeters N/10 NaOH necessary to neutralize 100 c.c. of stomach contents. This is the method of calculation most widely used. For other forms of expressing total acidity see page 243. Plot your results in a form similar to those shown in Figs. 69 and 70.

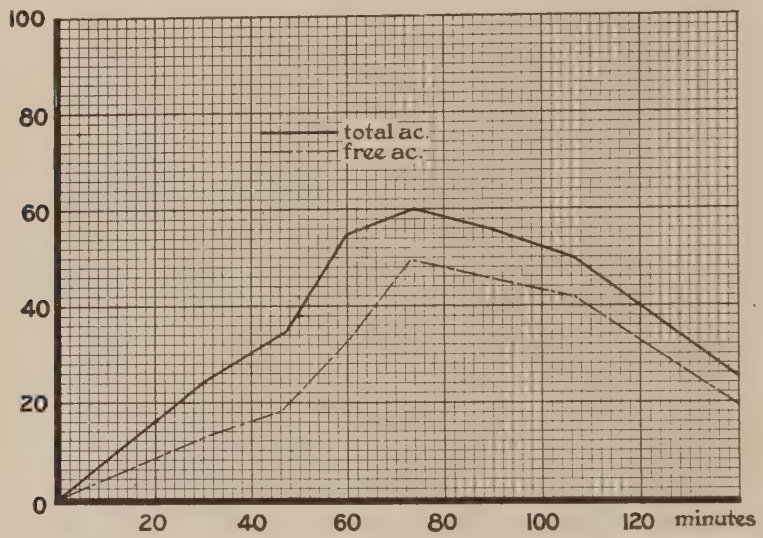


FIG. 69.—ACIDITY CURVES OF NORMAL HUMAN STOMACH.

Curves Obtained by the Fractional Method.—When an Ewald test meal is given to normal individuals a curve such as indicated below is usually obtained. The curve may vary within certain limits depending on individual idiosyncrasies, but is usually found to follow the curve depicted, and the meal normally leaves the stomach in two and one-half hours. Pathologically every variation occurs, both in time of evacuation as well as the character of the curve and the quantity of the

[1] *Procedure for Serial Titrations.*—When a series of titrations are to be made the following procedure may be used: Arrange the numbered evaporating dishes in rows on a tray. Introduce 1 c.c. of the proper sample into each dish, dilute with 10 c.c. of water and add the indicator. Add the N/100 NaOH to contents of dish No. 1 at a definite rate until a point is reached at which a faint pink color is obtained, as described above. Return dish No. 1 to its place in the tray and place dish No. 2 under the burette. Take the burette reading of No. 1. Then titrate No. 2 in the same way. Continue the series. This procedure has the advantage of being *speedy* and *accurate*. There is a slight error made by the rapid addition of the NaOH but it is *uniform* and the results (titrations) are therefore comparable.

secretion elaborated. Fig. 62 represents some of the possibilities of pathological cases, but a consideration of their interpretation is outside the purpose of the present volume. It will be evident, however, from a consideration of the figure that the cycle of gastric digestion is a constantly changing one, and no information concerning the trend of digestion can be obtained by an examination of only a single stage of digestion. Marked changes may precede or follow that stage and the possibilities suggested in Fig. 62 are all observed clinically and are of varying significance. Typical curves from cases of hyperacidity, gastric carcinoma and achylia are shown in Figs. 70, 71 and 72 respectively.

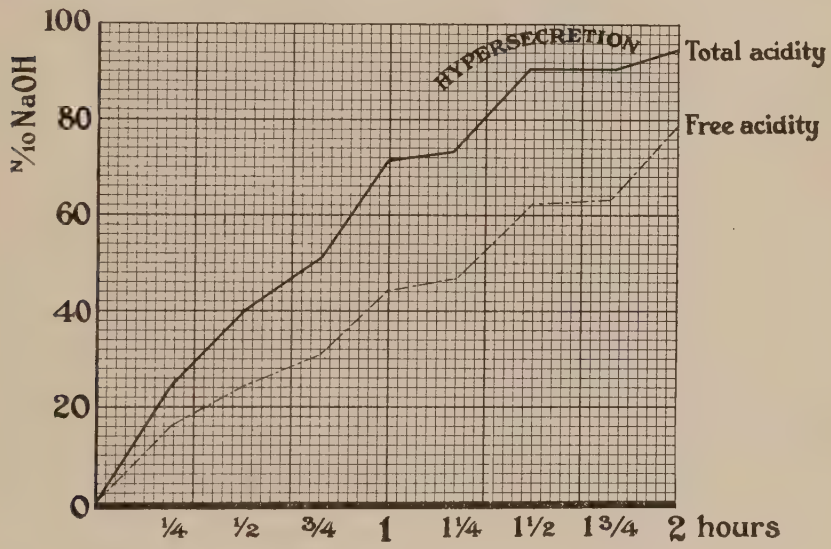


FIG. 70.—ACIDITY CURVES FROM A CASE OF HYPERSECRETION.

(b) Determination of Free Acidity.—The reagent most widely used, clinically, for the determination of free hydrochloric acid in stomach contents is Töpfer's reagent (see page 243). It has been found, however that this reagent gives rather inaccurate results due to the uncertain end point. Michaelis suggests that in using this indicator it should be titrated to a *salmon pink* and not to a *yellow* color as is ordinarily done.[1] A sharper end-point may be obtained with Sahli's reagent[2] which contains KI and KIO_3 and liberates iodine in the presence of free hydrochloric acid. The liberated iodine is titrated by thiosulphate using starch as an indicator. It gives values similar to Töpfer's reagent in

[1] Michaelis: "*Practical Physical and Colloid Chemistry*," Cambridge, 1925.

[2] A mixture of equal parts of a 48 per cent solution of potassium iodide and an 8 per cent solution of potassium iodate.

average acidities.[1] Acidities other than free hydrochloric react to a certain extent with Sahli's reagent, so that, for example, high results are obtained after the ingestion of acid fruits.

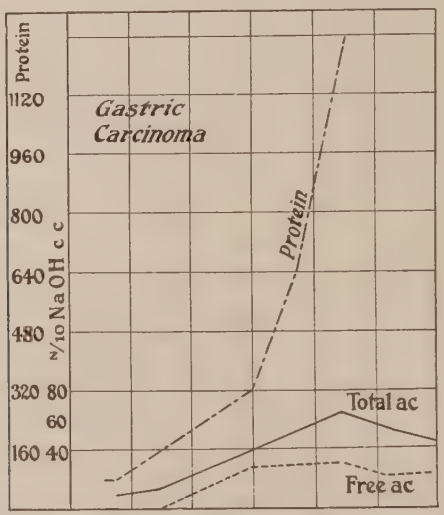


FIG. 71.—ACIDITY AND PROTEIN CURVES IN GASTRIC CARCINOMA. (*Clarke and Rehfuss: Jour. Am. Med. Ass'n*, 64, 1737, 1915.)

Procedure.—Measure 1 c.c. of the strained stomach contents by means of an Ostwald pipette and introduce it into a 60 c.c. porcelain evaporating dish. Dilute with 10 c.c. of distilled water, and add 1 c.c. of Sahli's reagent (a mixture of equal parts of 48 per cent KI and 8 per cent KIO_3). Allow the stomach contents

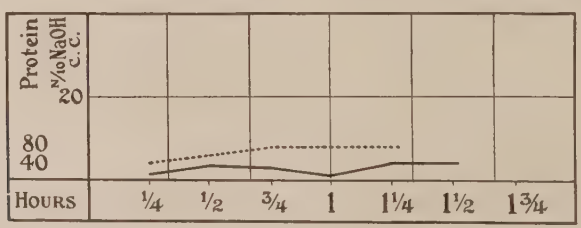


FIG. 72.—TOTAL ACIDITY AND PROTEIN CURVES IN BENIGN ACHYLIA (SOLID LINE REPRESENTS ACIDITY). (*Clarke and Rehfuss: Jour. Am. Med. Ass'n*, 64, 1737, 1915.)

thus treated to stand for five minutes and then titrate with N/100 sodium thiosulphate until only a faint yellow color remains. Now add 5–10 drops of a 1 per cent solution of soluble starch and continue the titration until the blue color disappears. In serial titrations the same procedure may be employed as described on page 230, note 1.

Calculation.—Note the number of cubic centimeters of N/100 sodium thiosulphate required to titrate 1 c.c. of stomach contents to the total disappearance of blue color in the presence of starch. Inasmuch as N/100 thiosulphate is

[1] Fowler, Bergeim and Hawk: Unpublished data.

PLATE IV.

COLOR NUANCES OF STANDARD SOLUTIONS CONTAINING 0.02 C.C. OF 0.2 PER CENT. ALCOHOLIC THYMOL SULPHONEPHTHALEIN (THYMOL BLUE) PER C.C. OF SOLUTION, AND ALSO THE CORRESPONDING VALUE IN TERMS OF pH AND IN C.C. OF NORMAL HYDROCHLORIC ACID PER 100 C.C. OF GASTRIC CONTENTS. (Shohl and King—*Johns Hopkins Hospital Bulletin*, 31, 158, 1920.)

equivalent to N/100 alkali, this value indicates the number of cubic centimeters of N/100 sodium hydroxide necessary to neutralize the free hydrochloric acid in 1 c.c. of the stomach contents. Multiply the value by 10 to obtain the number of cubic centimeters of N/10 NaOH necessary to neutralize 100 c.c. of stomach contents. This is the method of calculation most widely used. For other forms of expressing free acidity see page 243. Plot your results in a curve similar to those shown in Figs. 67, 69, and 70, pages 220, 230 and 231.

(c) Determination of Hydrogen Ion Concentration of Gastric Contents.—*Principle.*—Inasmuch as it is the hydrogen ion concentration of the gastric contents which is of importance for peptic digestion the determination of pH is coming into wider use in gastric analysis. Simple colorimetric methods are available for this purpose. They give information of real value and the test paper method especially is so convenient that it may often be the method of choice. Determinations of pH do not, however, displace titration methods as the latter give additional information.

(1) Colorimetric Method of Shohl and King.[1]—Procedure.—Prepare Clark and Lubs standard solutions for pH 1.4 to 3.0, especially standards for pH 1.4, 1.6, 1.8, 2.0, 2.4, 3.0. These are prepared as follows (see Chapter I): To 5 c.c. portions of 0.2 M potassium chloride (14.912 gm. of the dried recrystallized salt per liter) add the following quantities of 0.2 N HlC and dilute to 20 c.c.: pH 1.4, 4.15 c.c.; pH 1.8, 1.66 c.c.; pH 2.0, 1.06 c.c. To 5 c.c. portions of 0.2 M acid potassium phthalate (40.828 gm. of the dried recrystallized salt per liter) add the following quantities of 0.2 N HCl and dilute to 20 c.c.: pH 2.4, 3.96 c.c; pH 3.0, 2.03 c.c. To these standard solutions indicator is then added in the same proportion as is used for the clear gastric juice. Transfer 2 c.c. of filtered or centrifuged gastric contents to a test tube 11 mm. in diameter. Add 2 drops or 0.04 c.c. of 0.2 per cent thymolsulphonephthalein in 95 per cent alcohol. Compare with equal amounts of standard solutions treated in the same way in similar test tubes. (Plate IV opposite.) If the sample is more acid than pH 1.6 add 2 c.c. of water and two drops more of indicator and multiply reading by 2. Using the Ewald test meal it may be said in a general way that pH 1.4 represents a high acidity, from 1.6–1.8 moderate normal acidity, 2.0–2.4 low acidity and 3.0 and over anacidity.

(2) Method of Denis and Silverman Using Test Papers.[2]—Procedure.—Prepare test papers as follows. A solution of dye (dimethylaminoazobenzol 0.5 per cent or 0.1 per cent thymol blue) is poured over a sheet of filter paper six inches in diameter, the excess dye allowed to drain off and the paper dried quickly by holding it in front of a rapidly revolving electric fan. Prepare the two kinds of paper and cut in strips approximately 1 × ¼ inch and place in a stoppered bottle. Keep away from light and laboratory fumes. Highest grade smooth, ashless quantitative paper should be used.

[1] Shohl and King: *Bull. Johns Hopkins Hosp.*, 31, 158, 1920.

[2] Denis and Silverman: *Am. J. Med. Sci.*, 169, 25, 1925.

A complete outfit for this method may be obtained from the La Motte Chemical Products Company, Baltimore, Md.

Pour some of the material to be tested over the end of a test paper. The colors may be compared with those obtained from similar papers dipped in the standard solutions mentioned in method of Shohl and King above. However with some practice this will be unnecessary. All readings should be made at once. The table given below will serve as a guide in interpretation of results. It gives test paper results as compared with number of c.c. N/10 NaOH that would be required to neutralize 100 c.c. of a solution of the H ion concentration found, if all of the acid were free HCl.

Indicator	pH	Color	C.c. 0.1 N NaOH required	Remarks
Töpfer's reagent	1.4	Deep red	44	Hyperacidity.
Töpfer's reagent	1.6	Reddish-orange	27	Normal acidity.
Töpfer's reagent	1.8	Orange	17	Normal acidity.
Töpfer's reagent	2.0	Yellowish-orange	10	Hypoacidity.
Thymol blue	2.0	Purple-red	10	Hypoacidity.
Thymol blue	2.4	Faint pink	4	Hypoacidity.
Thymol blue	3.0	Yellow	1	Anacidity.

(d) Determination of Peptic Activity.—(1) Method of Mett[1] as Modified by Nirenstein and Schiff.[2]—*Principle.*—Small glass tube filled with coagulated egg albumin are introduced into the solution to be tested, and kept for a definite length of time in the incubator. The protein column is digested at both ends of the tube to an extent depends ing upon the amount of pepsin present. The method is not strictly accurate but is the most satisfactory for clinical purposes on account of its simplicity. Nirenstein and Schiff showed that human gastric juice contained inhibiting substances the effect of which is overcome by the dilution recommended.

Procedure.—Introduce into a small Erlenmeyer flask 1 c.c. of gastric juice and 15 c.c. of N/20 HCl (= 0.18 per cent HCl). Add two Mett tubes prepared as indicated below, stopper the flask to prevent evaporation and place in an incubator at 37°C. for 24 hours. By means of a low power microscope and a millimeter scale (graduated to half millimeters) determine accurately the length of the column of albumin digested at each end of the tubes. It is well to run the determination in duplicate in which case the result is the average of the eight figures obtained. Ordinariiy from 2–4 mm. of albumin are digested by normal human gastric juice.

Calculation.—The peptic power is expressed as the square of the number of millimeters of albumin digested. This is based on the Schütz-Borissow law that the amount of proteolytic enzyme present in a digestion mixture is proportional to the square of the number of millimeters of albumin digested. Therefore a

[1] Mett: *Arch. f. Anat. u. Physiol., Verda* 1894, 68.
[2] Nirenstein and Schiff: *Arch. f. ankheruungsitekn,* 8, 559, 1902.

gastric juice which digests 2 mm. of albumin contains four times as much pepsin as one which digests only 1 mm. of albumin.

Example.—If the microscopic reading gives on an average 2.2 mm. of albumin digested the pepsin value for the diluted juice would be $2.2^2 = 4.84$, and for the pure undiluted juice, $4.84 \times 16 = 77.44$.

Preparation of Mett Tubes (*Christiansen's Method*).[1]—The liquid portions of the whites of several eggs are mixed and strained through cheese cloth. The mixture should be homogeneous and free from air bubbles. It is best to allow the egg-white to stand for two or three hours in a vacuum desiccator to more completely remove air. A number of thin-walled glass tubes of 1–2 mm. internal diameter are thoroughly cleaned and dried and cut into lengths of about 10 inches. These are sucked full of the egg-white and kept in a horizontal position. Into a large evaporating dish or basin 5–10 liters of water are introduced and heated to boiling. The vessel is then removed from the fire and stirred with a thermometer until the temperature sinks to exactly 85°C. The tubes filled with egg-white are immediately introduced and left in the water until it has cooled. The tubes thus prepared are soft boiled, more easily digested than hard boiled tubes, and free from air bubbles. The ends are sealed by dipping in melted paraffin or sealing wax (preferably the latter), and the tubes can be kept thus for a long time. When ready for use mark with a file and break into pieces about ¾ inch long. After cutting, the tubes should be immediately introduced into the digestion mixture or may be kept a short time under water. Tubes whose ends are not squarely broken off must be rejected.

The digestibility of different egg-whites varies widely. Hence in making up a new set of tubes if we wish our results to be comparable these tubes must be standardized against those first prepared. This may be done by running simultaneous tests with tubes from the two series, using the same gastric juice and comparing the lengths of the columns digested in each case. Christiansen's method of preparing tubes of the same digestibility is to be preferred. He proceeds as in the original preparation of the tubes except that as the water cools from 90°–80°C. a single tube containing the new egg-white is dropped in at each degree change of temperature, that is at 90°, 89°, etc. Pieces of each of these tubes as well as of the original standard tubes are then allowed to digest simultaneously in portions of the same gastric juice. One of these tubes should show a digestibility equal to that of the standard tubes. For example the tube coagulated at 88°C. may show the proper digestibility. Then the new series of tubes should be made in the same manner as this one, that is introduced at 88°C. The tubes thus prepared should be again checked up with the standard to see that no mistake has been made.

(2) **Rose's Modification[2] of the Jacoby-Solms Method.**[3]—Dissolve 0.25 gram of the globulin of the ordinary garden pea,[4] *Pisum sativum*, in 100 c.c. of 10 per cent

[1] Christiansen: *Biochem. Zeit.*, 46, 257, 1912.
[2] Rose: *Archives of Internal Medicine*, 5, 459, 1910.
[3] Solms: *Zeitschrift für klinische Medizin*, 64, 159, 1907.
[4] The globulin may be prepared as follows: "The finely ground peas, freed as much as possible from the outer coating, are repeatedly extracted with large quantities of 10 per cent sodium chloride solution, the extracts combined, strained through fine bolting-cloth, and allowed to stand over night in large cylinders to deposit insoluble matter. The supernatant fluid is siphoned off and saturated with ammonium sulphate. The precipitate of albumin and globulin is filtered off, suspended in a little water, and dialyzed in running water for three days, until the salt has been removed, and the albumins have been dissolved. The globulins are filtered off and washed two or three times to remove the last trace of albumins. To purify further, the precipitate is extracted with 10 per cent sodium chloride solution, and

sodium chloride solution, warming slightly if necessary.[1] Filter and introduce 1 c.c. of the clear filtrate into each of a series of six[2] test-tubes about 1 cm. in diameter. Introduce into each tube 1 c.c. of 0.6 per cent hydrochloric acid and permit a period of about five minutes to elapse for the development of the turbidity. Make a known volume of the gastric juice (5–10 c.c. is sufficient) exactly neutral to litmus paper with dilute alkali; and record the volume of the alkali so used. If acid metaprotein precipitates, filter it off; if there is no precipitate proceed without filtration. Dilute the clear neutral solution with a known quantity of distilled water (usually 5 volumes) making proper allowance for the volume of alkali used in the neutralization. Boil 5–10 c.c. of the diluted juice, filter and add the following decreasing volumes (c.c.) to the series of six tubes: 1.0, 0.9, 0.7, 0.5, 0.2, 0.0. Make the measurements by means of a 1 c.c. pipette graduated in 0.01 c.c. Now rapidly introduce the *unboiled*, diluted juice in the following increasing volumes (c.c.) in order: 0.0, 0.1, 0.3, 0.5, 0.8, 1.0. Each tube now contains a total volume of 3 c.c. and a total acidity of 0.2 per cent hydrochloric acid. Shake each tube thoroughly and place them at 50–52°C. for 15 minutes or at 35–36°C. for one hour. Examine the series of tubes at the end of the digestion period and select that tube which contains the smallest quantity of gastric juice and which *shows no turbidity*. The volume of the juice used in this tube is taken as the basis for the calculation of the peptic activity.

Calculation.—The peptic activity is expressed in terms of 1 c.c. of the *undiluted* juice. For example, if it requires 0.5 c.c. of the diluted juice (five-fold dilution) to clear up the turbidity in 1 c.c. of the globulin solution in the proper experimental time interval (15 minutes or one hour according to temperature) the peptic activity would be expressed as follows:

$$(1 \div 0.5) \times 5 = 10 \text{ (peptic activity).}$$

According to this scale of pepsin units 10 may be considered as "normal" peptic activity. These units are about $\frac{1}{10}$ as large as those expressed by the Jacoby-Solms scale.

Inasmuch as it has been shown[3] that blood serum contains an antipepsin it is said to be advisable to test the gastric juice for blood before determining its proteolytic power. However, Dezani[4] claims that the methods for demonstrating antipepsin in the blood are not adequate.

(3) **Given's Modification of Rose's Method.**[5]—The gastric contents are strained through cheese cloth. Two c.c. are measured by means of an Ostwald pipette into a 25 c.c. stoppered volumetric cylinder, and diluted to the mark with distilled water. Into each of seven small test-tubes (1×10 cm.) is measured, with an Ostwald pipette, 1 c.c. of a 0.25 per cent filtered pea globulin in 10 per cent

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filtered until perfectly clear. The resulting solution is neutralized to litmus paper by the cautious addition of dilute sodium hydroxide, and again dialyzed in running water for three days to remove the salts completely. The precipitated globulins are then filtered off and dried on a water-bath at 40°C. During the entire process of separation the proteins should be preserved with a mixture of alcoholic thymol and toluol." This dried globulin is used in the clinical procedure.

[1] This solution may be preserved at least two months under toluene.

[2] A longer series of tubes may be used if desired. However, experience has shown that a series of six ordinarily affords sufficient range for all diagnostic purposes.

[3] Oguro: *Biochemische Zeitschrift*, 22, 266, 1909.

[4] Dezani: *Arch. farm. Oper.*, 22, 287, 1916.

[5] Givens: *Hygienic Lab. Bull.* 101, p. 71, August, 1915.

sodium chloride solution. To each tube is added 1 c.c. of 0.6 per cent hydrochloric acid, also by means of an Ostwald pipette. The tubes are allowed to stand about five minutes, until the maximum turbidity develops. To the first five, distilled water is added as follows: To the first, 0.9 c.c.; to the second, 0.8 c.c.; to the third, 0.7 c.c.; to the fourth, 0.6 c.c.; and to the fifth, 0.2 c.c.; to the sixth and seventh, none. Then there are rapidly added to each test-tube the following amounts of the diluted (1:12.5) gastric juice; to the first, 0.1 c.c.; to the second, 0.2 c.c.; to the third, 0.3 c.c.; to the fourth, 0.5 c.c.; to the fifth, 0.8 c.c.; to the sixth, 1.0 c.c.; and to the seventh, 1.0 c.c. of the diluted juice boiled. These measurements can be accurately made with a 1 c.c. pipette graduated in 0.01 c.c. All tubes are then immersed for 15 minutes in a water-bath at 50° to 52°C. At the end of this time, the tube is selected which is clear and contains the least amount of diluted gastric juice. Upon this basis, the peptic activity is calculated as the number of cubic centimeters of 0.25 per cent globulin digested by 1 c.c. of undiluted gastric juice. For example, if tube 2 containing 0.3 c.c. of a 12.5 times diluted juice be clear, then the result would be expressed:

$$\text{Peptic activity} = (1 \div 0.3) \times 12.5 = 41.2.$$

Ordinarily this scheme of seven tubes is used, though it is not a rule. If the free acidity be high, sometimes a dilution of $\frac{1}{25}$ is made. The number of tubes used will depend upon the accuracy desired.

(e) **Determination of Tryptic Activity.**—Trypsin is not a gastric enzyme but occurs in the pancreatic juice (see page 256). In case of regurgitation of intestinal contents through the pylorus trypsin would be passed into the stomach. This regurgitation is doubtless of frequent occurrence and may even be a normal mechanism by which gastric acidity is regulated (see page 220). Trypsin is, therefore, generally present in the contents of the normal human stomach. Inasmuch, however, as trypsin is destroyed by the pepsin-hydrochloric acid of the gastric juice, determinations of this enzyme must be carried out immediately after aspirations of the gastric contents, particularly where the acidity of the latter is high.

Spencer's Method.[1]—(*a*) Prepare five reagent tubes, Nos. 1, 2, 3, 4, and 5; more if desired.

To tubes 1 and 2 add 0.5 c.c. of gastric contents (filter if cloudy).

(*b*) To tubes 2, 3, 4, and 5 add 0.5 c.c. of distilled water.

(*c*) From tube 2 remove 0.5 c.c. of its mixed contents and add to tube 3. Mix thoroughly and add 0.5 c.c. from tube 3 to tube 4. Repeat for tube 5.

We now have dilutions of gastric contents of 1, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, and $\frac{1}{16}$.

(*d*) To each tube add one drop of phenolphthalein solution (phenolphthalein 1 gram; alcohol (95 per cent) 100 c.c.); then add drop by drop a 2 per cent sodium carbonate solution until a light pink color is produced.

[1] Elaborated by Dr. W. H. Spencer (*Jour. Biol. Chem.*, 21, 165, 1915) in the author's laboratory for the specific purpose of determining trypsin in gastric juice. For other trypsin methods see Chapter XI.

(*e*) To tubes 1, 2, 3, and 4 add 0.5 c.c. of casein solution. Tube 5 must receive 1 c.c. of casein solution, since it contains 1 c.c. of the diluted gastric contents. For the casein solution, dissolve 0.4 gram of casein in 40 c.c. of N/10 NaOH. Add 130 c.c. of distilled water, then 30 c.c. of N/10 HCl. This leaves the solution alkaline to the extent of 10 c.c. of N/10 NaOH, minus about 3 c.c. neutralized by the casein.

(*f*) Incubate for five hours at 40°C.

(*g*) Precipitate the undigested casein by dropwise addition of a solution of the following composition: glacial acetic acid 1 c.c., alcohol (95 per cent) 50 c.c., distilled water 50 c.c. The tubes in which digestion has been complete remain clear; others become turbid.

(*h*) The tryptic values are expressed in terms of dilution. Thus, complete digestion in tube 3 (a dilution of ¼) shows four times the tryptic power of undiluted gastric juice; taking this dilution as unity, the undiluted juice has a tryptic value of 4.

(*i*) Controls of boiled gastric contents plus casein solution, and of distilled water plus casein solution, treated as above stated, must show no digestion, and become turbid on addition of the precipitating solution.

(f) **Detection of Lactic Acid.**—When the acidity of the stomach contents is reduced to a low value there may occur considerable fermentation of carbohydrates which have been introduced into the stomach in the ingested food. This fermentation yields various organic acids among which lactic acid is particularly prominent. It is important, therefore, in case of low gastric acidity that the stomach contents be examined for lactic acid.

Tests. 1. Ether-Ferric Chloride Test (Strauss).—A satisfactory deduction regarding the presence of lactic acid can only be made by removing the lactic acid from disturbing factors (*e.g.*, hydrochloric acid, protein digestion products, etc.) present in the stomach contents. Lactic acid may be extracted from the stomach contents by ether. The following technic not only serves to detect lactic acid but also gives an approximate idea as to the amount of the acid present.

Procedure.—Introduce 5 c.c. of strained stomach contents into a small graduated separatory funnel, add 20 c.c. of ether and shake the mixture thoroughly. Permit the ether to separate, then allow all the fluid to run out of the separatory funnel except the upper 5 c.c. of ether. To this ether extract add 20 c.c. distilled water and 2 drops of a 10 per cent solution of ferric chloride and shake the mixture gently. A slight green color is obtained in the presence of 0.05 per cent lactic acid whereas 0.1 per cent lactic acid yields a very intense yellowish-green color.

2. Ferric Chloride Test (Kelling).—Fill a test-tube with water, add 1–2 drops of a 10 per cent solution of ferric chloride and mix thoroughly to form a liquid which is very faintly colored. Divide the solution into two parts and keep one part as a control. To the other part add a small amount of the strained gastric contents and to the control tube add a similar volume of water. Lactic acid is indicated by the immediate development of a distinct yellow color in the tube containing the gastric contents.

The color in this test is due to the formation of ferric lactate.

3. Uffelmann's Reaction.—Prepare 25 c.c. of Uffelmann's reagent.[1] To 5 c.c. of this reagent in a test-tube add an equal volume of strained gastric juice. A canary yellow or greenish-yellow color develops if lactic acid be present to the extent of 0.01 per cent or over.

Other organic acid gives a similar reaction. Mineral acids such as hydrochloric acid discharge the blue coloration leaving a *colorless* solution. In other words, the color of the reagent is weakened in the presence of an *acid* reaction.

(g) Detection of Occult Blood.[2]—1. Benzidine Reaction.—This is one of the most delicate of the reactions for the detection of blood. Different benzidine preparations vary greatly in their sensitiveness, however. Inasmuch as benzidine solutions change readily upon contact with light it is essential that they be kept in a dark place. The test is performed as follows: To 3 c.c. of a saturated solution of benzidine in glacial acetic acid[3] add 2 c.c. of the solution to be tested and then 1 c.c. of 3 per cent hydrogen peroxide. A positive test is indicated by a green or blue color.

Confirmatory test.—If the mixture contains fat make neutral or slightly alkaline and extract by shaking with an equal volume of ether. Discard this ether extract. Make the residue acid with acetic acid and again extract with ether. Pour off ether into a small evaporating dish. Put on a hot water bath with flame turned out and evaporate to dryness. Add a few drops of water, a drop of benzidine solution and a drop of 3 per cent hydrogen peroxide. A green or blue color indicates blood.

The sensitiveness of the benzidine reaction is greater when applied to aqueous solutions than when applied to the urine. (For further discussion of this test see chapter on Blood.) Blood is found in gastric contents in conditions associated with erosion of the mucous membrane, ulcer and carcinoma. In cases of ulcer the blood may be bright red or may be converted to brown acid hematin by the excess of acid which is usually present. In carcinoma the blood forms brownish black lumps, the so-called "coffee ground" material.

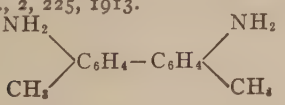
2. Ortho-tolidin Test (Ruttan and Hardisty).[4]—To 1 cc. of a 4 per cent solution of o-tolidin[5] in glacial acetic acid add 1 c.c. of the gastric juice under exam-

[1] Uffelmann's reagent is prepared by adding ferric chloride solution to a 1 per cent solution of carbolic acid until an amethyst-blue color is obtained, due in part to the formation of a ferric salt of carbolic acid and in part to the reduction of some of the iron.

[2] These tests may be made upon the strained stomach contents or upon the solid residue.

[3] Glacial acetic acid is preferable, but if not available, alcohol acidified with acetic acid may be used.

[4] Ruttan and Hardisty: *Canadian Medical Ass'n. Journal*, Nov., 1912; also *Biochem. Bull.*, 2, 225, 1913.

[5] 

ination and 1 cc. of 3 per cent hydrogen peroxide. In the presence of blood a bluish color develops (sometimes rather slowly) and persists for some time (several hours in some instances).

This test is said to be as sensitive for the detection of occult blood in feces and stomach contents as is the benzidine reaction. It is also claimed to be more satisfactory for urine than any other blood test. The acetic acid solution may be kept for one month with no reduction in delicacy.

(h) **Detection of Bile in Stomach Contents.**—If we accept Boldyreff's theory as to the automatic regulation of gastric acidity[1] under normal conditions by the regurgitation of alkaline material from the intestine, then the presence of bile in the gastric juice does not possess the clinical significance it has been accorded. However, if an ordinary Ewald meal be fed, and bile in any considerable quantity be found throughout the entire course of digestion it may indicate, pathologically, a stenosis below the level of the common bile duct. Frequently samples of gastric contents are encountered which are uncolored and which nevertheless contain bile. It is also true that bile may be adsorbed from stomach contents by mucus and food rests. The regulation technic for bile testing is often inadequate to demonstrate the presence of this fluid in gastric contents. The following procedure based upon the oxidation of the bilirubin with nitric acid forming green biliverdin is delicate and easy of application.

Procedure.—Saturate 10 c.c. of the fluid portion of the stomach contents with powdered ammonium sulphate. This may be accomplished by shaking for one to two minutes. It generally requires about 1 inch of powdered sulphate in the bottom of an ordinary test-tube to obtain full saturation. When the fluid is saturated add 1–3 c.c. of acetone and thoroughly mix the contents of the tube by inverting the tube five or six times. (It is better not to shake.) Permit the tube to stand and allow the acetone to rise to the surface. This acetone contains the bile pigment if any is present in the stomach contents. Allow a drop of yellow nitric acid to flow down the side of the tube and note the green color in the acetone.

This green color is biliverdin which has been produced from the bilirubin by oxidation with nitric acid. If too much acid is added the green color will be oxidized to a purple or red. If the acetone does not rise to the surface promptly the liquid has not been completely saturated with ammonium sulphate.

If the stomach contents contains large amounts of bile as indicated by a deep green color, 4–5 drops of the fluid may be diluted with 10 c.c. water and the above test applied.

[1] Boldyreff: *Quart. Jour. Exp. Med.*, 8, 1, 1914.

(i) **Microscopy of the Gastric Contents.**—A microscopical examination of the gastric contents is a routine clinical procedure.

When an Ewald meal is given the starch granules in various stages of digestion are observed together with epithelia from the pharynx, esophagus, and occasionally the stomach. Gastric and salivary mucus are seen and readily recognized by their ropy appearance. Pathologically various bacteria are seen, sarcinæ, Oppler-Boas bacilli,

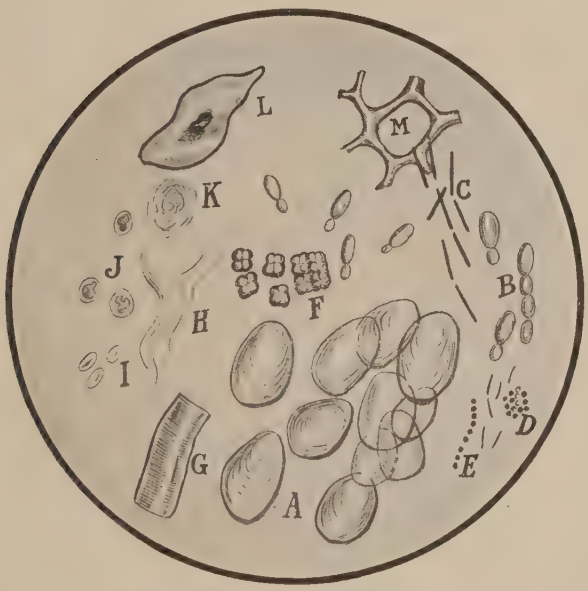


FIG. 73.—MICROSCOPICAL CONSTITUENTS OF THE GASTRIC CONTENTS.

A, Starch cells; *B*, yeast cells; *C*, Oppler-Boas bacilli; *D*, staphylococci; *E*, streptococci; *F*, sarcinæ; *G*, muscle fibre; *H*, mucus; *I*, red blood cells; *J*, leucocytes; *K*, snail-like mucus formations; *L*, squamous epithelial cell; *M*, cellulose.

streptococci, leptothrix, etc. Retained food from previous meals is readily recognized by its histological appearance; meat fibers, vegetable cells, and cellulose may all occur in pathological retention. In certain pathological processes such as ulcer and cancer, red blood cells, pus, and even the cancer cells themselves may be found. For illustrations of the microscopical constituents of gastric contents, see Fig. 73.

Procedure.—Examine a drop of the original (mixed) stomach contents unstained under the low and high powers of the microscope. Compare your findings with the microscopical views shown in Fig. 55.

Wolff Technic for the Protein Concentration of the Gastric Contents.[1]—Owing to the diagnostic importance of the protein concentration of the gastric secretion, a short note of this test is given here. Under normal conditions the protein concentration follows that of acidity rather closely. In certain cases, however, such as carcinoma (Fig. 53), there is an actual increase in the protein concentration of the gastric juice out of all proportion to the acidity. The test may be made as follows: The regular Ewald test meal is fed and specimens of the gastric contents are obtained at 15-minute intervals by means of the Rehfuss tube. One c.c. of the filtered juice is then diluted with 9 c.c. of water representing a dilution of 1:10; 5 c.c. of this mixture is again added to 5 c.c. of water and a dilution of 1:20 obtained; this is again repeated using 5 c.c. of the mixture last obtained and 5 c.c. of distilled water and the dilutions are kept up until a series is obtained representing 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, and if necessary 1:640 or more. They are then stratified with approximately 1 c.c. of Wolff's reagent,[2] care being taken that the liquids do not mix. The tubes should be read immediately against a dark background and the tube giving a protein ring at the greatest dilution of gastric juice recorded. The curve in Fig. 71 shows a pronounced case of gastric carcinoma. With normal acid figures the protein concentration evolves proportionally to the acidity. A case of achylia is shown in Fig. 72.

Töpfer's Method of Gastric Analysis

This method is much less elaborate than many others but is sufficiently accurate for ordinary clinical purposes. The method embraces the volumetric determination of (1) *total acidity*, (2) *free acidity* (*organic and inorganic*),[3] and (3) *free hydrochloric acid*, and the subsequent calculation of (4) *combined acidity* and (5) *acidity due to organic acids and acid salts*, from the data thus obtained.

Procedure.—Feed the Ewald test meal as directed on page 228. At the end of *one hour* remove the *entire stomach contents* and analyze as directed below. This method of procedure is less accurate than the Fractional Method (see page 217). Measure the volume of the gastric contents, strain it through cheese cloth and introduce 10 c.c. of the strained material into each of three small beakers or porcelain dishes.[4] Label the vessels *A*, *B*, and *C*, respectively, and proceed with the analysis according to the directions given below. The volume of fluid present in the stomach one hour after an Ewald meal varies under normal conditions between 50 and 100 c.c. In cases of hypersecretion or defective motility 200–300 c.c. may be found. Very excessive volumes, *e.g.*, 500–3000 c.c., are indicative of dilatation of the stomach and suggest pyloric stenosis, either benign or malignant.

[1] Wolff: Magen- und Darmkrankh., Berlin, 1912, p. 217; also *Berl. klin. Woch.*, May 29, 1911, and March 18, 1912.
Rolph: *Med. Rec.*, 1913, p. 848.
Clarke and Rehfuss: *Jour. Am. Med. Ass'n*, 64, 1737, 1915.

[2] Phosphotungstic acid ..... 0.3 gm.
Concentrated hydrochloric acid ..... 1.0 c.c.
Alcohol 95 per cent ..... 20.0 c.c.
Distilled water sufficient to make ..... 200.0 c.c.

[3] For a discussion of *combined* acid see chapter on Gastric Digestion.

[4] If sufficient gastric juice is not available it may be diluted with water or a smaller amount, *e.g.*, 5 c.c., taken for each determination.

1. **Total Acidity.**[1]—Add 3 drops of a 1 per cent alcoholic solution of phenolphthalein[2] to the contents of vessel *A* and titrate with N/10 sodium hydroxide solution until a *faint pink* color is produced and persists for almost two minutes. Take the burette reading and calculate the total acidity.

Calculation.—The total acidity may be expressed in the following ways:

1. The number of cubic centimeters of N/10 sodium hydroxide solution necessary to neutralize 100 c.c. of gastric juice.

2. The weight (in grams) of sodium hydroxide necessary to neutralize 100 c.c. of gastric juice.

3. The weight (in grams) of hydrochloric acid which the total acidity of 100 c.c. of gastric juice represents, *i.e.*, percentage of hydrochloric acid.

The forms of expression most frequently employed are 1 and 3, preference being given to the former, particularly in clinical work.

In making the calculation note the number of cubic centimeters of N/10 sodium hydroxide required to neutralize 10 c.c. of the gastric juice and multiply it by 10 to obtain the number of cubic centimeters necessary to neutralize 100 c.c. of the fluid. If it is desired to express the acidity of 100 c.c. of gastric juice in terms of hydrochloric acid, by weight, multiply the value just obtained by 0.00365.[3]

2. **Free Acidity (Organic and Inorganic).**—Add 3 drops of sodium alizarin sulphonate solution[4] to the contents of vessel *B* and titrate with N/10 sodium hydroxide solution until a *violet* color is produced. In this titration the red color, which appears after the tinge of yellow due to the addition of the indicator has disappeared, must be entirely replaced by a *distinct violet color*. Take the burette reading and calculate the free acidity due to organic and inorganic acids.

Calculation.—Since the indicator used reacts to both organic and inorganic acids, the number of cubic centimeters of N/10 sodium hydroxide used indicates the free acidity of 10 c.c. of gastric juice. The data for 100 c.c. of gastric juice may be calculated according to the directions given under Total Acidity, see above.

3. **Free Hydrochloric Acid.**[5]—Add 4 drops of dimethylaminoazobenzene (Töpfer's reagent) solution[6] to the contents of the vessel *C* and titrate with N/10 sodium hydroxide solution until the initial red color is replaced by *salmon pink*.[7] Take the burette reading and calculate the free acidity.

Calculation.—The indicator used reacts only to free hydrochloric acid, hence the number of cubic centimeters of N/10 sodium hydroxide used indicates the volume necessary to neutralize the *free hydrochloric acid* of 10 c.c. of gastric juice. To determine the data for 100 c.c. of gastric juice proceed according to the directions given under Total Acidity, page 230.

4. **Combined Acidity.**—This value may be obtained by subtracting the number of cubic centimeters of N/10 sodium hydroxide used in neutralizing the contents of vessel *B* from the number of cubic centimeters of N/10 sodium hydroxide used in neutralizing *A*. The data for 100 c.c. of gastric juice may be calculated according to directions given under Total Acidity, page 230.

[1] This includes free and combined acid and acid salts.
[2] One gram of phenolphthalein dissolved in 100 c.c. of 95 per cent alcohol.
[3] One c.c. of N/10 hydrochloric acid contains 0.00365 gram of hydrochloric acid.
[4] One gram of sodium alizarin sulphonate dissolved in 100 c.c. of water.
[5] Hydrochloric acid *not* combined with protein material.
[6] One-half gram dissolved in 100 c.c. of 95 per cent alcohol.
[7] Michaelis: Practical Physical and Colloid Chemistry, Cambridge, 1925. If a yellow color appears as soon as the indicator is added it denotes the *absence of free* acid.

5. Acidity Due to Organic Acids and Acid Salts.—This value may be conveniently calculated by subtracting the number of cubic centimeters of N/10 sodium hydroxide used in neutralizing the contents of vessel *C* from the number of cubic centimeters of N/10 sodium hydroxide solution used in neutralizing the contents of vessel *B*. The remainder indicates the number of cubic centimeters of N/10 sodium hydroxide solution necessary to neutralize the acidity due to organic acids and acid salts present in 10 c.c. of gastric juice. The data for 100 c.c. of gastric juice may be calculated according to directions given under Total Acidity, page 230.

CHAPTER X

FATS[1]

FATS occur very widely distributed in the plant and animal kingdoms, and constitute the third general class of food-stuffs. In plant organisms they are to be found in the seeds, roots, and fruit, while each individual tissue and organ of an animal organism contains more or less of the substance. In the animal organism fats are especially abundant in the bone marrow and adipose tissue. They contain the same elements as the carbohydrates, *i.e.*, carbon, hydrogen, and oxygen, but the oxygen is present in smaller percentage than in the carbohydrates

FIG. 74.—BEEF FAT. (*Long.*)

and the hydrogen and oxygen are not present in the proportion to form water.

Chemically considered the fats are esters[2] of the tri-atomic alcohol, glycerol, and the mono-basic fatty acids. In the formation of these fats three molecules of water result. This water arises by the replacement of the H's of the carboxyl groups of the three fatty acid molecules by the glycerol radical, thus yielding the following type of formula. In this case the combination is with palmitic acid ($C_{15}H_{31}COOH$).

$$\begin{array}{l} CH_2\text{—}OOCC_{15}H_{31} \\ | \\ CH\text{ —}OOCC_{15}H_{31} \\ | \\ CH_2\text{—}OOCC_{15}H_{31} \end{array}$$

[1] For literature see Bloor: *Physiol. Reviews*, 2, 92, 1922: *Jour. Metab. Res.*, 4, 547, 1923 and *Chem. Rev.* 2, 243, 1925 also Allen: *Jour. Metab. Res.*, 2, 219, 1922: and Leathes and Raper: "The Fats," Longmans, Green & Co., London, 1925.

[2] An ester is an acid, one or more of whose acid hydrogens is replaced by an organic radical.

The three fatty acid radicals entering into the structure of a neutral fat may be the radicals of the same fatty acid or they may consist of the radicals of three different fatty acids.

By hydrolysis of a neutral fat, *i.e.*, by the addition to the molecule of those elements which are eliminated in the formation of the fat from glycerol and fatty acid, it may be resolved into its component parts, *i.e.*, glycerol and fatty acid. In the case of palmitin the following would be the reaction:

$$\underset{\text{Palmitin.}}{C_3H_5(OOCC_{15}H_{31})_3} + 3H_2O \rightarrow \underset{\text{Glycerol.}}{C_3H_5(OH)_3} + 3\underset{\text{Palmitic acid.}}{(C_{15}H_{31}COOH)}.$$

This process is called *saponification* and may be produced by boiling with alkalis; by the action of steam under pressure; by long-continued contact with air and light; by the action of certain bacteria and by fat-splitting enzymes or lipases, *e.g.*, *pancreatic lipase* (see page 258). The cells forming the walls of the intestines evidently possess the peculiar property of synthesizing the glycerol and fatty acid thus formed so that after absorption these bodies appear in the blood not in their individual form but as neutral fats.

The principal animal fats with which we have to deal are *stearin*, *palmitin*, *olein*, and *butyrin*. Such less important forms as laurin and myristin may occur abundantly in plant organisms. The older system of nomenclature for these fats was to apply the prefix "tri" in each case (*e.g.*, *tri*-palmitin) since three fatty acid radicals are contained in the neutral fat molecule.

The fatty acids corresponding to the above-mentioned animal fats are *stearic*, $CH_3(CH_2)_{16}COOH$; *palmitic*, $CH_3(CH_2)_{14}COOH$; *oleic*, $CH_3(CH_2)_7CH=CH(CH_2)_7COOH$; and *butyric*, $CH_3(CH_2)_2COOH$. Stearic, palmitic and butyric acids are *saturated* fatty acids, whereas oleic acid belongs to the class of *unsaturated* acids. Linoleic acid is also unsaturated. Upon the presence of these unsaturated fatty acids depends the property which certain fats possess of absorbing or combining with iodine. The determination of this so-called "iodine absorption number" is important in the differentiation of fats and oils. Fats containing the *unsaturated acids* oleic and linoleic may be transformed by "hydrogenation"[1] into the fats containing the corresponding *saturated acid* (stearic). The oleic acid is changed thus:

$$\underset{\text{Oleic acid.}}{C_{17}H_{33}COOH} + 2H \quad \underset{\text{Stearic acid.}}{C_{17}H_{35}COOH}.$$

Fats occur ordinarily as mixtures of several individual fats. For

[1] Addition of hydrogen to the molecule, producing a "hydrogenated fat."

example, the fat found in animal tissues is a mixture of olein, palmitin and stearin, the percentage of any one of these fats present depending upon the particular species of animal from whose tissue the fat was derived. Thus the ordinary mutton fat contains more stearin and less olein than the pork fat. Human fat contains from 67 per cent to 85 per cent of olein and, according to Benedict and Osterberg, upon analysis yields 76.08 per cent of carbon and 11.78 per cent of hydrogen. Butter consists in large part of olein and palmitin. Stearin, butyrin, caproin and traces of other fats are also present.

A synthetic fat known as *intarvin* contains an odd number of carbon atoms, consisting chiefly of the glycerol ester of margaric acid, $C_{17}H_{34}O_2$. It has been recommended[1] as a food fat for diabetics on the presumption that by the process of β-oxidation the formation of the harmful acetone bodies would not result. Its use has been criticized[2] however, on the ground that other acids, lactic and pyruvic, may be formed through α-oxidation, resulting in an acidosis. Like natural fats, intarvin seems to require carbohydrate for its complete utilization.

Pure neutral fats are odorless, tasteless, and generally colorless. They are insoluble in the ordinary protein solvents such as water, salt solutions, and dilute acids and alkalis, but are very readily soluble in ether, benzene, chloroform, and *boiling* alcohol. The neutral fats are non-volatile substances possessing a *neutral* reaction. If allowed to remain in contact with the air for a sufficient length of time they become yellow in color, assume an *acid* reaction and are said to be *rancid*. The neutral fats may be crystallized, some of them with great facility. The crystalline forms of some of the more common fats are reproduced in Figs. 74, 75 and 76 on pages 245, 248 and 251. Each individual fat possesses a specific melting-point, and this property of melting at a definite temperature may be used as a means of differentiation in the same way as the coagulation temperature (see page 169) is used for the differentiation for coagulable proteins. When shaken with water, or a solution of albumin, soap, or acacia, the liquid fats are finely divided and assume a condition known as an *emulsion*. The emulsion with water is transitory, while the emulsions with soap, acacia, or albumin are permanent.

The *vitamin* or accessory food substance known as "Fat-soluble A" is present in certain foods, *e.g.* milk, butter and egg yolk and in particularly large amount in cod liver oil. It is believed to be absent from lard, olive oil and certain other vegetable oils (see Chap. XXXII). Another vita-

[1] Kahn: *Am. Jour. Med. Sci.*, 166, 826, 1923.
[2] Lundin: *Jour. Metab. Res.*, 4, 151, 1923.
Modern: *Ibid.*, 4, 177, 1923.

min known as vitamin D (or the antirachitic factor) also occurs abundantly in cod liver oil, puffer fish oil[1] etc. (see Chapter XXXII).

The fat ingested continues essentially unaltered until it reaches the intestine where it is acted upon by *pancreatic lipase* (*steapsin*), the fat-splitting enzyme of the pancreatic juice (see page 258), and glycerol and fatty acid are formed. The glycerol is absorbed directly. The fatty acid thus formed unites with the alkalis of the pancreatic juice and forms soluble soaps. These soaps are readily absorbed. That bile is of assistance in the absorption of fat is indicated by the increase of fat in the feces when for any reason bile does not pass into the intestine. Bloor[2] claims that neither petroleum hydro-

FIG. 75.—MUTTON FAT. (*Long.*)

carbons nor nonsaponifiable esters, *e.g.*, wool fat (lanolin), are absorbed. He believes that *saponification is a necessary preliminary to absorption.*

It has been shown[3] that the common food fats are from 93 to 98 per cent utilized by the normal human body.

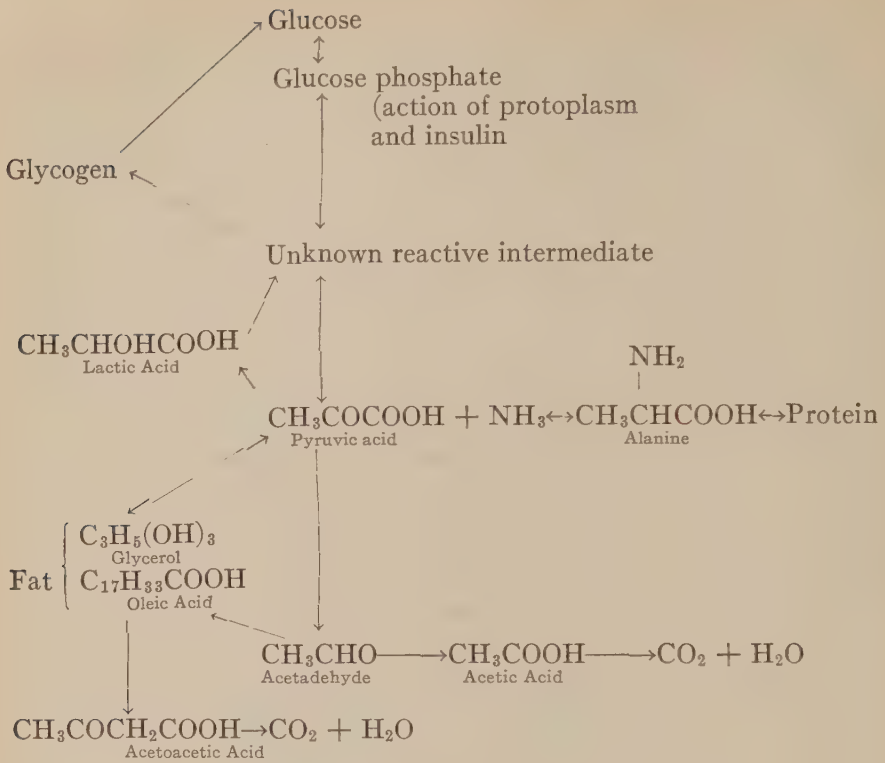
The fat distributed throughout the animal body is formed partly from the ingested fat and partly from carbohydrates and the "carbon moiety" of protein material. The formation of *adipocere*[4] and the occurrence of *fatty degeneration* are sometimes given as proofs of the formation of fat from protein. This is questioned by many investigators. Rather more satisfactory and direct proof of the formation of fat from protein material has been obtained by Hofmann in experimentation with *fly-maggots*. The normal content of fat in a number of maggots was determined and later the fat content of others which had developed

[1] Hess and Weinstock: Report before *Soc. Exp. Biol. Med.*, March, 1926.
[2] Bloor: *Jour. Biol. Chem.*, 15, 105, 1913.
[3] Holmes and Deuel: *Jour. Biol. Chem.*, 41, 227, 1920.
[4] For a very complete analysis of adipocere see Ruttan and Marshall: *Jour. Biol Chem.* 29, 319, 1917.

in blood (84 per cent of the solid matter of blood plasma is protein material) was determined. The fat content was found to have increased 700 to 1100 per cent as a result of the diet of blood proteins. The celebrated experiments of Pettenkofer and Voit, however, have furnished what is, perhaps, the most substantial positive evidence of the formation of fat from protein. These investigators fed dogs large amounts of lean meat, daily, and through examination of urine, feces and expired air were enabled to account for *only part of the ingested carbon*, although obtaining a satisfactory nitrogen balance. The discrepancy in the carbon balance was explained upon the theory that the protein of the ingested meat had been split into a *nitrogenous* and a *non-nitrogenous* portion in the organism, and that the non-nitrogenous portion, the so-called "carbon moiety" of the protein, had been subsequently transformed into fat and deposited as such in the tissues of the organism. Later evidence in favor of the formation of fat from protein has been furnished by the experiments of Weinland.[1] This investigator worked with the larvæ of *Calliphora*,[2] these larvæ being rubbed up in a mortar[3] with Witte's peptone and water to form a homogeneous mixture. After placing these mixtures at 38°C. for 24 hours the fat content was found to have increased, as much as 140 per cent in some instances. The active agency in this transformation of fat is the *larval tissue*, since the tissues of both the dead and living larvæ possess the property. Data are given from control tests which show that the action of bacteria in this transformation of protein was excluded. Lusk[4] as the result of experiments on dogs claims to have definitely demonstrated that fat may be formed from protein.

The diagram given on page 250[5] indicates possible intermediate steps in the transformation of carbohydrates into fats and proteins (and *vice versa*) in the animal body.

[1] Weinland: *Zeit. f. Biol.*, 51, 197, 1908.
[2] The ordinary "blow-fly."
[3] Intact larvæ were used in some experiments.
[4] Atkinson and Lusk: *Proc. Nat. Acad. Sci.*, 5, 246, 1919; Lusk: *Proc. Soc. Expt. Biol. and Med.*, 17, 171, 1920.
Atkinson, Rapport and Lusk: *Jour. Biol. Chem.*, 53, 155, 1922.
[5] Adapted from Shaffer: *Physiological Reviews*, 3, 394, 1923, and Mathews: *Physiological Chemistry*, Fourth Edition, New York, 1925.



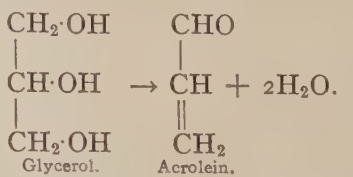
Experiments on Fats

1. Solubility.—Test the solubility of olive oil in water, dilute acid and alkali and in cold alcohol, hot alcohol, chloroform, ether, and carbon tetrachloride.

2. Formation of a Transparent Spot on Paper.—Place a drop of olive oil upon a piece of ordinary writing paper. Note the transparent appearance of the paper at the point of contact with the fat.

3. Reaction.—Try the reaction of fresh olive oil to litmus, Congo red and phenolphthalein. Repeat the test with rancid olive oil.[1] What is the reaction of a fresh fat and how does this reaction change upon allowing the fat to stand for some time?

4. Formation of Acrolein.—To a little olive oil in a mortar add some dry potassium bisulphate, $KHSO_4$, and rub up thoroughly. Transfer to a dry test-tube and cautiously heat. Note the irritating odor of acrolein. The glycerol of the fat has been dehydrated and acrylic aldehyde or acrolein has been produced. This is the reaction which takes place:



[1] To prepare *rancid* olive oil add 5 drops of oleic acid to 10 c.c. of olive oil.

5. Emulsification.—(a) Shake up a drop of neutral[1] olive oil with a little water in a test-tube. The fat becomes finely divided, forming an emulsion. This is not a permanent emulsion since the fat separates and rises to the top upon standing.

(b) To 5 c.c. of water in a test-tube add 2 or 3 drops of 0.5 per cent Na_2CO_3. Introduce into this faintly alkaline solution a drop of neutral olive oil and shake. The emulsion while not permanent is not so transitory as in the cases of water free from sodium carbonate.

(c) Repeat (b) using rancid olive oil. What sort of an emulsion do you get and why? It is impossible to emulsify a highly rancid fat due to the excessive formation of rather insoluble soaps about the oil drops.

(d) Shake a drop of neutral olive oil with dilute albumin solution. What is the nature of this emulsion? Examine it under the microscope.

6. Fat Crystals.—Dissolve a small piece of lard in ether in a test-tube, add an equal volume of alcohol and allow the alcohol-ether mixture to evaporate spontaneously. Examine the crystals under the microscope and compare them with those reproduced in Figs. 74, 75, and 76, on pages 245, 248 and 251.

7. Saponification of Bayberry Tallow.[2]—Fill a large casserole two-thirds full of water rendered strongly alkaline with solid potassium hydroxide (a stick one inch in length). Add about 10 grams of bayberry tallow and boil, keeping the volume constant by adding water as needed. When saponification is com-

FIG. 76.—PORK FAT.

plete[3] remove 25 c.c. of the soap solution for use in Experiment 8 and add concentrated hydrochloric acid slowly to the remainder until no further precipitate is produced.[4] Cool the solution and the precipitate of free fatty acid will rise to the surface and form a cake. In this instance the fatty acid is principally palmitic acid. Remove the cake, break it into small pieces, wash it with water by decantation and transfer to a small beaker by means of 95 per cent alcohol. Heat on a

[1] Neutral olive oil may be prepared by shaking ordinary olive oil with a 10 per cent solution of sodium carbonate. This mixture should then be extracted with ether and the ether removed by evaporation. The residue is *neutral* olive oil.

[2] Bayberry tallow is derived from the fatty covering of the berries of the *wax myrtle*. It is therefore frequently called "myrtle wax" or "bayberry wax."

[3] Place 2 or 3 drops in a test-tube full of water. If saponification is complete the products will remain in solution and no oil will separate.

[4] Under some conditions a purer product is obtained if the soap solution is cooled before precipitating the fatty acid.

water-bath until the palmitic acid is dissolved, then filter through a dry filter paper and allow the filtrate to cool slowly in order to obtain satisfactory crystals. Write the reactions which have taken place in this experiment.

When the palmitic acid has completely crystallized filter off the alcohol, dry the crystals between filter papers and try the tests given in Experiment 10, below.

8. Salting-out Experiments.—To 25 c.c. of soap solution, prepared as described above, add solid sodium chloride to the point of saturation, with continual stirring. A menstruum is thus formed in which the soap is insoluble. This salting-out process is entirely analogous to the salting-out of proteins (see page 169).

9. Formation of Insoluble Soaps.—Introduce 5 c.c. of soap solution into each of two test-tubes. To the contents of one tube add a small amount of a solution of calcium chloride and to the contents of the other tube add a small amount of a solution of magnesium sulphate. Note the formation of insoluble soaps of calcium and magnesium.

10. Palmitic Acid.—(a) Examine the crystals under the microscope and compare them with those shown in Fig. 77, below.

(b) Solubility.—Try the solubility of palmitic acid in the same solvents as used on fats (see page 250).

(c) Melting-point.—Determine the melting-point of palmitic acid by the method given on page 253.

FIG. 77.—PALMITIC ACID.

(d) Formation of Translucent Spot on Paper.—Melt a little of the fatty acid and allow a drop to fall upon a piece of ordinary writing paper. How does this compare with the action of a fat under similar circumstances?

(e) Acrolein Test.—Apply the test as given under 4, page 250. Explain the result.

(f) Iodine Absorption Test.—For directions see Experiment 13.

11. Saponification of Lard.—To 25 grams of lard in a flask add 75 c.c. of alcoholic-potash solution[1] and warm upon a water-bath until saponification is complete. (This point is indicated by the complete solubility of a drop of the solution when allowed to fall into a little water.) Now transfer the solution from the flask to an evaporating dish containing about 100 c.c. of water and heat on a water-bath until all the alcohol has been driven off. Precipitate the fatty acid with hydrochloric acid and cool the solution. Remove the fatty acid which rises

[1] A 20 per cent solution in 40 per cent alcohol may be used.

to the surface,[1] neutralize the solution with sodium carbonate and evaporate to dryness. Extract the residue with alcohol, remove the alcohol by evaporation upon a water-bath and on the residue of glycerol thus obtained make the tests as given below.

12. Glycerol. (a) Taste.—What is the taste of glycerol?

(b) Solubility.—Try the solubility of glycerol in water, alcohol and ether.

(c) **Hypochlorite-Orcinol Reaction.**[2]—This is based on the oxidation of glycerol to the corresponding aldose sugar glycerose and the detection of the latter by means of orcinol. Homologues of glycerol as well as the corresponding acids and certain sugars as glucose and mannose give the reaction. The first named occur seldom while the latter may be removed with baryta.

Two to 3 c.c. of a 1 per cent or 0.1 per cent solution of glycerol in water are treated with exactly 3 drops (= 0.12 c.c.) normal NaOCl[3] and boiled for a minute. To the liquid while still hot add 3 drops of hydrochloric acid (sp. gr. 1.124) and boil 30–60 seconds to drive off chlorine, a colorless solution being obtained. Then add an equal volume of fuming hydrochloric acid and a small knife-point of orcinol. On boiling the mixture becomes a beautiful violet or green blue. The precipitate formed is soluble in amyl alcohol and may be examined spectroscopically.

(d) Acrolein Test.—Repeat the test as given under 4, page 250.

(e) Borax Fusion Test.—Fuse a little glycerol on a platinum wire with some powdered borax and note the characteristic green flame. This color is due to the glycerol ester of boric acid.

(f) Benedict's Test.—How does this result compare with the results on the sugars?

(g) Solution of $Cu(OH)_2$. Form a little cupric hydroxide by mixing copper sulphate and potassium hydroxide. Add a little glycerol to this suspended precipitate and note what occurs.

13. Iodine Absorption Test.—Dissolve a small amount of an unsaturated organic acid, e.g., oleic acid, in chloroform. Add 2–3 drops of Hübl's iodine solution[4] and shake. The solution will be decolorized if unsaturated acids are present. This is due to the absorption of the iodine. The test should be controlled by shaking chloroform and iodine solution to which no acid has been added.

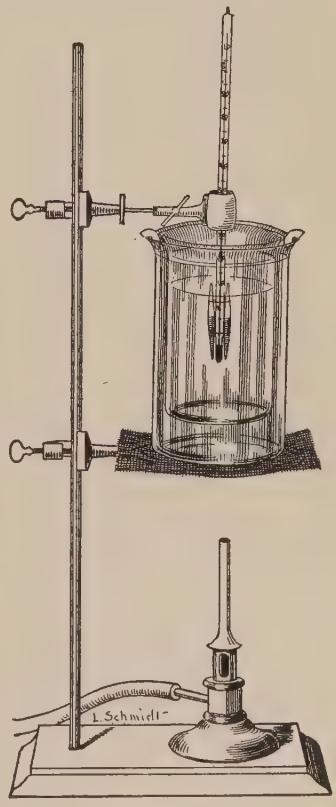


FIG. 78.—MELTING-POINT APPARATUS.

14. Melting-point of Fat.—Procedure.—

[1] After drying the acid make an *iodine absorption test* as described in Experiment 1.

[2] Mandel and Neuberg: *Bioch. Zeit.*, 71, 214, 1915.

[3] Made according to Raschig: *Ber.*, 40, 4586, 1907.

[4] Prepared by dissolving 26 grams of iodine and 30 grams of mercuric chloride in one litre of 95 per cent alcohol.

Insert one of the melting-point tubes, furnished by the instructor, into the liquid fat and draw up the fat until the bulb of the tube is about one-half full of the material. Then fuse one end of the tube in the flame of a Bunsen burner and fasten the tube to a thermometer by means of a rubber band in such a manner that the bottom of the fat column is on a level with the bulb of the thermometer (Fig. 78, p. 253). Fill a beaker of medium size about two-thirds full of water and place it within a second larger beaker which also contains water, the two vessels being separated by pieces of cork. Immerse the bulb of the thermometer and the attached tube in such a way that the bulb is about midway between the upper and the lower surfaces of the water of the inner beaker. The upper end of the tube being open it must extend above the surface of the surrounding water. Apply gentle heat, stir the water, and note the temperature at which the fat first begins to melt. This point is indicated by the initial transparency. For ordinary fats, raise the temperature very cautiously from 30°C. To determine the congealing-point remove the flame and note the temperature at which the fat begins to solidify. Record the melting- and congealing-points of the various fats submitted by the instructor.

15. Surface Tension Lowering Property of Fat.—Perform the experiment described in Chapter I, p. 22.

CHAPTER XI

PANCREATIC DIGESTION[1]

As soon as the food mixture leaves the stomach it comes into intimate contact with the bile and the pancreatic juice. Since these fluids are alkaline in reaction (see Bile, page 273) there can obviously be little further peptic activity after they have become intimately mixed with the chyme and have neutralized most of the acidity previously imparted to it by the hydrochloric acid of the gastric juice. The pancreatic juice reaches the intestine through the duct of Wirsung which opens into the intestine near the pylorus.

Normally the secretion of pancreatic juice is brought about by the stimulation produced by the acid chyme as it enters the duodenum. Therefore, any factor which produces an increased flow of gastric juice such, for example, as water[2] will cause a stimulation of the pancreatic secretion. The secretion of pancreatic juice is probably not due to a nervous reflex as was believed by Pawlow but rather, as Bayliss and Starling have shown, is dependent upon the presence, in the epithelial cells of the duodenum and jejunum of a body known as *prosecretin.* This body is changed into *secretin* through the hydrolytic action of the acid present in the chyme. The secretin is then absorbed by the blood, passes to the pancreas and stimulates the pancreatic cells, causing a flow of pancreatic juice. The quantity of juice secreted under these conditions is proportional to the amount of secretin present. The activity of secretin solutions is not diminished by boiling, hence the body does not react like an enzyme. Further study of the body may show it to be a definite chemical individual of relatively low molecular weight. It has not been possible thus far to obtain secretin from any tissues except the mucous membrane of the duodenum and jejunum.

This *secretin* mentioned above belongs to the class of substances called *hormones* or chemical messengers. These hormones play a very important part in the coordination of the activities of certain functions and glands. Other important hormones are those elaborated by the thyroids, parathyroids, the adrenals, the pituitary body (hypophysis), the embryo and the reproductive glands. It is claimed by some that all active organs of the body produce hormones. (See Chapter XX on Endocrine Organs.)

[1] Under this head we will consider only such digestive processes as are brought about by enzymes originating in the pancreas. In the following chapter on Intestinal Digestion will be found a consideration of such enzymes as have a true *intestinal origin.*

[2] See chapter on Gastric Digestion.

The juice as obtained from a permanent fistula differs greatly in its properties from the juice as obtained from temporary fistula, and neither form of fluid possesses the properties of the normal fluid. Pancreatic juice collected by Glaessner from a natural fistula has been found to be a colorless, clear, strongly alkaline fluid which foams readily. It is further characterized by containing albumin, globulin, proteose, and peptone; nucleoprotein is also present in traces.[1] The average daily secretion of pancreatic juice is 650 c.c. and its specific gravity is 1.008. The fluid contains 1.3 per cent of solid matter and the freezing-point is $-0.47°C$. The normal pancreatic secretion contains at least five distinct enzymes. They are *trypsin*, a proteolytic enzyme; *pancreatic amylase* (amylopsin), an amylolytic enzyme; *pancreatic lipase* (steapsin), a fat-splitting enzyme; *pancreatic rennin*, a milk-coagulating enzyme and *erepsin* a peptide-splitting enzyme.[2]

The most important of the five enzymes of the pancreatic juice is the proteolytic enzyme *trypsin*. This enzyme resembles pepsin in so far as each has the power of breaking down protein material, but the trypsin has much greater digestive power and is able to cause a more complete decomposition of the complex protein molecule. In the process of normal digestion the protein constituents of the diet are for the most part transformed into proteoses (albumoses) and peptones before coming in contact with the enzyme trypsin. This is not absolutely essential, however, since trypsin possesses digestive activity sufficient to transform unaltered native proteins and to produce from their complex molecules comparatively simple fragments. Among the products of tryptic digestion are *proteoses*, *peptones*, *peptides*. Through the action of erepsin these are broken down to simple amino acids[2] such as *leucine*, *tyrosine*, *aspartic acid*, *glutamic acid*, *alanine*, *phenylalanine*, *glycocoll*, *cystine*, *serine*, *valine*, *proline*, *oxyproline*, *isoleucine*, *arginine*, *lysine*, *histidine*, and *tryptophane*. (The crystalline forms of many of these products are reproduced in Chapter V.) We thus see that trypsin has no action on simple peptides but does set free some amino acids from casein for example.[2] This is apparently done by splitting them from longer peptide chains. Trypsin does not occur preformed in the gland, but exists there as a zymogen called *trypsinogen* which bears the same relation to trypsin that pepsinogen does to pepsin. Trypsin has never been obtained in a pure form and therefore very little can be stated definitely as to its nature. The enzyme is the most active in alkaline solution (pH 8 to 9) but is also active in neutral or slightly acid solutions. Trypsin is destroyed by mineral acids and may also be

[1] Glaessner: *Zeitschrift für physiologische Chemie*, 40, 476, 1904.

[2] Waldschmidt-Leitz and Harteneck: *Zeit. physiol. Chem.*, 147, 286, 1925: 149, 203 and 221, 1925.

destroyed by comparatively weak alkali (2 per cent sodium carbonate) if left in contact for a sufficiently long time. It is also inactivated by x-ray.[1] Trypsinogen, on the other hand, is more resistant to the actions of alkalis. In pancreatic digestion the protein does not swell as is the case in gastric digestion, but becomes more or less "honey-combed" and finally disintegrates.

The presence of active *pepsin* in the contents of the intestine has been demonstrated by Abderhalden and Meyer.[2] It may possibly be that pepsin may play a part in the profound intestinal proteolysis which has up to this time been assigned to trypsin and erepsin (see chapter on Gastric Digestion).

The pancreatic juice which is collected by means of a fistula possesses practically no power to digest protein matter. A body called *enterokinase* occurs in the intestinal juice and has the power of converting trypsinogen into trypsin. This process is known as the "activation" of trypsinogen and through it a juice which is incapable of digesting protein may be made active. (For further discussion of enterokinase see chapter on Intestinal Digestion.) Mendel and Rettger[3] and others have demonstrated that activation of trypsinogen into trypsin may be brought about in the gland as well as in the intestine of the living organism. The manner of the activation in the gland and the nature of the body causing it are unknown at present. Prym[4] denies that such an activation occurs. After the death of the animal at least part of the trypsinogen in the pancreas is changed to trypsin as shown by the fact that the extract of the gland is active.

Delezenne claims that trypsinogen may be activated by soluble *calcium salts*. He reports experiments which indicate that proteolytically inactive pancreatic juice, obtained directly from the duct, when treated with salts of this character, assumes the property of digesting protein material. This process by which the trypsinogen is activated through the instrumentality of calcium salts is very rapid and is designated by Delezenne as an "explosion." The suggestion of Mays that there may possibly be several precursors of trypsin one of which is activated by enterokinase and the others by other agents, is of interest in this connection.

Boldyreff[5] has demonstrated the presence of trypsin in the stomach due to the regurgitation of duodenal contents through the pylorus

[1] Clark and Northrop: *Jour. Gen. Physiol.*, 9, 87, 1925.
[2] Abderhalden and Meyer: *Zeit. physiol. Chem.*, 74, 67, 1911.
[3] Mendel and Rettger: *American Journal of Physiology*, 7.
[4] Prym: *Pflüger's Archiv*, 104 and 107.
[5] Boldyreff: Transactions of the 11th Pirogoff's Congress of Physicians, St. Petersburg, 1910.

(see pages 216, 220 and 237). Others[1] have confirmed this finding (see Chapter IX on Gastric Analysis).

Pancreatic amylase (*amylopsin*), the second of the pancreatic enzymes, is an amylolytic enzyme which possesses somewhat greater digestive power than the salivary amylase (ptyalin) of the saliva. As its name implies, its activity is confined to the starches, and the products of its amylolytic action are dextrins and sugar. The sugar is principally maltose and this, by the further action of an inverting enzyme (maltase), is transformed into glucose.

It is possible that the saliva as a digestive fluid is not absolutely essential. The salivary amylase (ptyalin) is destroyed by the hydrochloric acid of the gastric juice and is therefore inactive when the chyme reaches the intestine. Should undigested starch be present at this point, however, it would be quickly transformed by the active pancreatic amylase. This enzyme is not present in the pancreatic juice of infants during the first few weeks of life, thus showing very clearly that a starchy diet is not normal for this period.

The pronounced influence of electrolytes upon the action of pancreatic amylase and other amylases has been demonstrated many times.[2] In fact the removal of electrolytes from pancreatic juice by dialysis yields a juice which possesses *no power to split starch.* It also appears that the Cl, Br and NO_3 ions have an important stimulating action upon the amylases.[3] The most active amylase thus far prepared has been described by Sherman and collaborators.[4]

It has been shown that pancreatic amylase will digest raw starch. The raw starch of corn and wheat may be completely digested and absorbed by normal adults whereas the raw potato starch is about 80 per cent available.[5]

The extent to which amylase is present in the feces has been taken as the index of pancreatic activity.

The third enzyme of the pancreatic juice is called *pancreatic lipase* (*steapsin*) and is a fat-splitting enzyme. It has the power of splitting the neutral fats of the food by hydrolysis, into fatty acid and glycerol. A typical reaction would be as follows:

$$\underset{\text{Palmitin.}}{C_3H_5(OOCC_{15}H_{31})_3} + 3H_2O \rightarrow 3(\underset{\text{Palmitic acid.}}{C_{15}H_{31}COOH}) + \underset{\text{Glycerol.}}{C_3H_5(OH)_3}.$$

Recent researches make it probable that fats undergo saponification prior to their absorption. The fatty acids formed unite with the

[1] Spencer, Meyer, Rehfuss and Hawk: *American Jour. Physiol.*, 39, 459, 1916

[2] For the literature see Kendall and Sherman: *Jour. Am. Chem. Soc.*, 32. 1087, 1910.

[3] Wohlgemuth: *Biochem. Zeit.*, 9, 10, 1908; and Kendall and Sherman: *Jour. Am. Chem. Soc.*, 32, 1087, 1910. Bierry: *Biochem. Zeit.*, 40, 357, 1912. Rockwood: *Jour. Am. Chem. Soc.*, 41, 228, 1919.

[4] See p. 56 and *Proc. Soc. Expt. Biol. and Med.*, 23, 413, 1926.

[5] Langworthy and Deuel: *Jour. Biol. Chem.*, 42, 27, 1920.

alkalis of the pancreatic juice and intestinal secretion to form soluble soaps which are readily absorbed. It was formerly believed that the fats could also be absorbed in emulsion—a condition promoted by the presence of the soluble soaps. After absorption the fatty acids are resynthesized to form neutral fats with glycerol.

Bloor[1] has reported experiments which "make it extremely probable that fats can be absorbed only in water-soluble form and that saponification is a necessary preliminary to absorption." Petroleum hydrocarbons and non-saponifiable esters, *e.g.*, *wool fat* (lanolin) were unabsorbed. Bloor further claims that in the absorption of fats there is a tendency toward the formation of a uniform chyle fat, presumably the characteristic body fat of the animal.

Pancreatic lipase is very unstable and is easily rendered inert by the action of acid. For this reason it is not possible to prepare an extract having a satisfactory fat-splitting power from a pancreas which has been removed from the organism for a sufficiently long time to have become acid in reaction.

The fourth enzyme of the pancreatic juice is called *pancreatic rennin*. It is a milk-coagulating enzyme whose action is very similar to that of the *gastric rennin* found in the gastric juice. It is supposed to show its greatest activity at a temperature varying from 60° to 65°C.

For a discussion of *erepsin*, the fifth pancreatic enzyme, see p. 266.

PREPARATION OF AN ARTIFICIAL PANCREATIC JUICE[2]

After removing the fat from the pancreas of a pig or sheep, finely divide the organ by means of scissors and grind it in a mortar. If convenient, the use of an ordinary meat chopper is a very satisfactory means of preparing the pancreas.

When finely divided as above the pancreas should be placed in a 500 c.c. flask, about 150 c.c. of 30 per cent alcohol added and the flask and contents shaken frequently for 24 hours. (What is the reaction of this alcoholic extract at the end of this period, and why?) Strain the alcoholic extract through cheese cloth, filter, nearly neutralize with potassium hydroxide solution and then exactly neutralize it with 0.5 per cent sodium carbonate.

Products of Pancreatic Digestion of Protein

Introduce into a 250 c.c. flask 20 grams of casein, 10 c.c. of the artificial pancreatic juice prepared as described above and 100 c.c. of 1 per cent sodium carbonate. Allow to digest at 40°C. for 8 to 10 days with the addition of a few cubic centimeters each of chloroform and toluene, the flask being stoppered with cotton. As the chloroform and toluene evaporate they must be renewed. Heat the mixture to boiling and at the boiling-point add acetic acid drop by drop until the mixture is acid in reaction. Cool and filter.

[1] Bloor: *Physiol. Reviews.* 2, 92, 1922.

[2] For other methods of preparation see Karl Mays: *Zeitschrift für physiologische Chemie*, 38, 428, 1903.

To five c.c. of the filtrate add bromine water drop by drop. Note the development of pink color which disappears in the presence of an excess of the reagent. This reaction indicates the presence of tryptophane.[1]

To another 5 c.c. portion of the filtrate add 10 drops of concentrated sulphuric acid and 10 c.c. of a 10 per cent solution of mercuric sulphate in 5 per cent sulphuric acid. After mixing and allowing to stand for a few minutes filter off the yellow precipitate which forms. This is a mercury compound of tryptophane.[2] Filter off the precipitate reserving the filtrate and wash the precipitate on the filter paper thoroughly with several small portions of water.

To small portions of the precipitate apply the Hopkins-Cole, xanthoproteic, and Millon tests. Tryptophane gives a positive reaction with the first two of these tests being responsible for the Hopkins-Cole reaction as applied to protein (see Chapter VI).

Test portions of the filtrate from the mercuric precipitate by the Hopkins-Cole, xanthoproteic, and Millon reactions. Tyrosine responds to the latter two tests.

To the remainder of the filtrate add a few drops of ammonia[3] and evaporate to a volume of 10 to 20 c.c. using at first a free flame and completing the evaporation on a water-bath. Transfer to a beaker and allow to stand for 1 or 2 days. Examine microscopically the crystals which separate out. Tyrosine crystallizes in sheaves of needles (see Fig. 46). Leucine forms small rosettes. Apply Mörner's reaction for tyrosine (see p. 150).

GENERAL EXPERIMENTS ON PANCREATIC DIGESTION

Experiments on Trypsin[4]

1. The Most Favorable Reaction for Tryptic Digestion.—Prepare seven tubes as follows:

(a) 2–3 c.c. of neutral pancreatic extract + 2–3 c.c. of water.

(b) 2–3 c.c. of neutral pancreatic extract + 2–3 c.c. of 1 per cent sodium carbonate.

(c) 2–3 c.c. of neutral pancreatic extract + 2–3 c.c. of 0.5 per cent sodium carbonate.

(d) 2–3 c.c. of neutral pancreatic extract + 2–3 c.c. of 0.2 per cent hydrochloric acid.

(e) 2–3 c.c. of neutral pancreatic extract + 2–3 c.c. of 0.2 per cent combined hydrochloric acid.

(f) 2–3 c.c. of neutral pancreatic extract + 2–3 c.c. of 0.4 per cent boric acid.

(g) 2–3 c.c. of neutral pancreatic extract + 2–3 c.c. of 0.4 per cent acetic acid.

Add a small piece of fibrin[5] to the contents of each tube and keep them at 40°C. noting the progress of digestion. In which tube do we find the most satisfactory

[1] Kurajeff: *Zeit. physiol. Chem.*, 36, 501, 1898–99.

[2] It has been claimed that a similar yellow precipitate forms in the presence of *tyrosine*, cystine and polypeptides. For *quantitative* estimation of tryptophane see Homer: *Jour. Biol. Chem.*, 22, 369, 1915.

[3] If the solution is alkaline in reaction, while it is being concentrated, the amino acids will be broken down and ammonia will be liberated.

[4] For these experiments as well as for those on the other pancreatic enzymes commercial preparations of *trypsin* and *pancreatin* may be employed.

[5] *Congo red fibrin* may be used in this and the following tests on tryptic digestion. If used the experiments should be made at room temperature. For preparation of this fibrin see Chapter II.

digestion, and why? How do the indications of the digestion of fibrin by trypsin differ from the indications of the digestion of fibrin by pepsin?

2. The Most Favorable Temperature.—(For this and the following series of experiments under tryptic digestion use the neutral extract plus an equal volume of 0.5 per cent sodium carbonate.) In each of four tubes place 5 c.c. of alkaline pancreatic extract. Immerse one tube in cold water from the faucet, keep a second at room temperature and place a third in the incubator or water-bath at 40°C. Boil the contents of the fourth for a few moments, then cool and also keep it at 40°C. Into each tube introduce a small piece of fibrin and note the progress of digestion. In which tube does the most rapid digestion occur? What is the reason?

3. Influence of Bile.—Prepare three tubes as follows:

(a) 5 c.c. of pancreatic extract + 0.5–1 c.c. of bile.

(b) 5 c.c. of pancreatic extract + 5 c.c. of bile.

(c) 5 c.c. of pancreatic extract.

Introduce into each tube a small piece of fibrin and keep them at 40°C. Shake the tubes frequently and note the progress of digestion. Does the presence of bile retard tryptic digestion? How do these results agree with those obtained under gastric digestion?

4. Quantitative Determination of Tryptic Activity.[1]—Gross' Method.—This method is based upon the principle that faintly alkaline solutions of casein are precipitated upon the addition of dilute (1 per cent) acetic acid whereas its digestion products are not so precipitated. The method follows: Prepare a series of tubes each containing 10 c.c. of a 0.1 per cent solution of pure, fat-free casein,[2] which has been heated to a temperature of 40°C. Add to the contents of the series of tubes increasing amounts of the trypsin solution under examination,[3] and place them at 40°C. for fifteen minutes. At the end of this time remove the tubes and acidify the contents of each with a few drops of dilute (1 per cent) acetic acid. The tubes in which the casein is completely digested will remain clear when acidified, while those tubes which contain undigested casein will become more or less turbid under these conditions. Select the first tube in the series which exhibits no turbidity upon acidification, thus indicating complete digestion of the casein, and calculate the tryptic activity of the enzyme solution under examination.

Calculation.—The unit of tryptic activity is an expression of the power of 1 c.c. of the fluid under examination exerted for a period of fifteen minutes on 10 c.c. of a 0.1 per cent casein solution. For example, if 0.5 c.c. of a trypsin solution completely digests 10 c.c. of a 0.1 per cent solution of casein in fifteen minutes, the activity of that solution would be expressed as follows:

$$\text{Tryptic activity} = 1 \div 0.5 = 2.$$

Such a trypsin solution would be said to possess an activity of 2. If 0.3 c.c. of the trypsin solution had been required the solution would be said to possess an activity of 3.3; i.e., $1 \div 0.3 = 3.3$.

[1] For a discussion of Spencer's method for the quantitative determination of trypsin in *stomach contents* see chapter on Gastric Analysis.

[2] Made by dissolving 1 gram of Grübler's casein in a liter of 0.1 per cent sodium carbonate. A little chloroform may be added to prevent bacterial action.

[3] The amount of solution used may vary from 0.1–1 c.c. The measurements may conveniently be made by means of a 1 c.c. graduated pipette.

Experiments on Pancreatic Amylase

1. The Most Favorable Reaction.—Prepare four tubes as follows:

(a) 1 c.c. of neutral pancreatic extract+1 c.c. of starch paste+2 c.c. of water.

(b) 1 c.c. of neutral pancreatic extract+1 c.c. of starch paste+2 c.c. of 1 per cent sodium carbonate.

(c) 1 c.c. of neutral pancreatic extract+1 c.c. of starch paste+2 c.c. of 0.5 per cent sodium carbonate.

(d) 1 c.c. of neutral pancreatic extract+1 c.c. of starch paste+2 c.c. of 0.2 per cent hydrochloric acid.

Shake each tube thoroughly and place them in the incubator or water-bath at 40°C. At the end of a half-hour divide the contents of each tube into two parts and test one part by the iodine test and the other part by Benedict's test. Where do you find the most satisfactory digestion? How do the results compare with those obtained from Experiment 1 under Trypsin, page 260?

2. The Most Favorable Temperature.—(For this and the following series of experiments upon pancreatic amylase use the neutral extract plus an equal volume of 0.5 per cent sodium carbonate.) In each of four tubes place 2–3 c.c. of alkaline pancreatic extract. Immerse one tube in cold water from the faucet, keep a second at room temperature, and place a third on the water-bath at 40°C. Boil the contents of the fourth for a few moments, then cool and also keep it at 40°C. Into each tube introduce 2–3 c.c. of starch paste and note the progress of digestion. At the end of one-half hour divide the contents of each tube into two parts and test one part by the iodine test and the other part by Benedict's test. In which tube do you find the most satisfactory digestion? How does this result compare with the result obtained in the similar series of experiments under Trypsin (see page 261)?

3. Influence of Bile.—Prepare three tubes as follows:

(a) 2–3 c.c. of pancreatic extract+2–3 c.c. of starch paste+0.5–1 c.c. of bile.

(b) 2–3 c.c. of pancreatic extract+2–3 c.c. of starch paste+5 c.c. of bile.

(c) 2–3 c.c. of pancreatic extract+2–3 c.c. of starch paste.

Shake the tubes thoroughly and place them in the incubator or water-bath at 40°C. Note the progress of digestion frequently and at the end of a half-hour divide the contents of each tube into two parts and test one part by the iodine test and the other part by Benedict's test. What are your conclusions regarding the influence of bile upon the action of pancreatic amylase?

4. Digestion of Dry Starch.—To a little dry starch in a test-tube add about 5 c.c. of pancreatic extract and place the tube in the incubator or water-bath at 40°C. At the end of a half-hour filter and test separate portions of the filtrate by the iodine and Benedict's tests. What do you conclude regarding the action of pancreatic amylase upon dry starch? Compare this result with that obtained in the similar experiment under Salivary Digestion (page 110).

5. Digestion of Inulin.—To 5 c.c. of inulin solution in a test-tube add 10 drops of pancreatic extract and place the tube in the incubator or water-bath at 40°C. After one-half hour test the solution by Benedict's test.[1] Is any reducing substance

[1] If the inulin solution gives a reduction before being acted upon by the pancreatic juice it will be necessary to determine the extent of the original reduction by means of a "check" test (see p. 96).

present? What do you conclude regarding the digestion of inulin by pancreatic amylase?

6. Quantitative Determination of Amylolytic Activity.—Wohlgemuth's Method.[1] Arrange a series of test-tubes with diminishing quantities of the enzyme solution under examination, introduce into each tube 5 c.c. of 1 per cent solution of soluble starch[2] and place each tube at once in a bath of ice-water.[3] When all the tubes have been prepared in this way and placed in the ice-water bath they are transferred to a water-bath or incubator and kept at 38°C. for from 30 minutes to an hour.[4] At the end of this digestion period the tubes are again removed to the bath of ice-water in order that the action of the enzyme may be stopped.

Dilute the contents of each tube, to within about 1/2 inch of the top, with water, add one drop of a N/10 solution of iodine and shake the tube and contents thoroughly. A series of colors ranging from dark blue through bluish violet and reddish yellow to yellow, will be formed.[5] The dark blue color shows the presence of unchanged starch, the bluish-violet indicates a mixture of starch and erythrodextrin, whereas the reddish-yellow signifies that erythrodextrin and maltose are present and the yellow solution denotes the complete transformation of starch into maltose. Examine the tubes carefully before a white background and select the last tube in the series which shows the entire absence of all blue color, thus indicating that the starch has been completely transformed into dextrins and sugar. In case of indecision between two tubes, add an extra drop of the iodine solution, and observe them again, after shaking.

Calculation.—The amylolytic activity[6] of a given solution is expressed in terms of the activity of 1 c.c. of such a solution. For example, if it is found that 0.02 c.c. of an amylolytic solution, acting at 38°C., completely transformed the starch in 5 c.c. of a 1 per cent starch solution in 30 minutes, the amylolytic activity of such a solution would be expressed as follows:

$$D\ {}^{38^\circ}_{30'} = 250$$

This indicates that 1 c.c. of the solution under examination possesses the power of completely digesting 250 c.c. of 1 per cent starch solution in 30 minutes at 38°C.

Wohlgemuth has suggested a slight alteration in the above procedure for use in the determination of the amylase content of the feces.[7] A modification of the Wohlgemuth procedure[8] for this purpose is given in Chapter XVI on Feces.

[1] Wohlgemuth: *Biochemische Zeitschrift*, 9, 1, 1908.

[2] For method preparing soluble starch, see p. 92. Kahlbaum's soluble starch is satisfactory. In preparing the 1 per cent solution, the weighed starch powder should be dissolved in *cold* distilled water in a casserole and stirred until a homogeneous suspension is obtained. The mixture should then be heated, with constant stirring, until it is clear. This ordinarily takes about 8–10 minutes. A slightly opaque solution is thus obtained which should be cooled and made up to the proper volume before using.

[3] Ordinarily a series of six tubes is satisfactory, the volumes of the enzyme solution used ranging from 1 c.c. to 0.1 c.c. and the measurements being made by means of a 1 c.c. graduated pipette. All tubes should contain the same volume of material. To accomplish this add appropriate amounts of distilled water to tubes receiving less than 1 c.c. of enzyme solution. Each tube should be placed in the ice-water bath as soon as the starch solution is introduced. It will be found convenient to use a small wire basket to hold the tubes.

[4] Longer digestion periods may be used where it is deemed advisable. If exceedingly weak solutions are being investigated, it may be most satisfactory to permit the digestion to extend over a period of 24 hours.

[5] See p. 106.

[6] Designated by "D" the first letter of "diastatic."

[7] Wohlgemuth: *Berliner klinische Wochenschrift*, 47, 92, 1910.

[8] Hawk: *Archives of Internal Medicine*, 8, 552, 1911.

Experiments on Pancreatic Lipase

1. Influence of Bile on Action of Lipase.—Prepare five test-tubes as follows:

(a) 5 c.c. neutral pancreatic extract + 0.5 c.c. olive oil + 4.5 c.c. water.

(b) 0.5 c.c. olive oil + 9.5 c c. water.

(c) 0.5 c.c. olive oil + 8.5 c.c. water + 1 c.c. bile.

(d) 5 c.c. neutral pancreatic extract + 0.5 c.c. olive oil + 3.5 c.c. water + 1 c.c. bile.

(e) 5 c.c. neutral pancreatic extract + 3.5 c.c. water + 1 c.c. bile.

Shake the tubes thoroughly, add a drop of toluene to each and place them in an incubator or water-bath at 40° for 24 hours. At the end of this period add a drop of phenolphthalein to each tube and titrate with N/20 NaOH to a permanent pink color. Shake the tube during the titration. Record the amount of N/20 alkali necessary to neutralize the contents of each tube. Which tube required the most? Why?

2. "Litmus-milk" Test.—Into each of two test-tubes introduce 10 c.c. of milk and a small amount of litmus solution.[1] To the contents of one tube add 3 c.c. of neutral pancreatic extract[2] and to the contents of the other tube add 3 c.c. of water or of boiled neutral pancreatic extract. Keep the tubes at 40°C. and note any changes which may occur. What is the result and how do you explain it?

3. Copper Soap Test for Lipase.[3]—Prepare a 2:100 agar-agar solution, mix with an equal volume of 5:100 starch paste, incorporate in this mass about 1/40 of its volume of the neutral fat desired (butter, lard, etc.), heat with constant agitation until a homogeneous emulsion is produced, pour into a Petri dish, and cool rapidly. Distribute on the surface of the solidified mass with a fine pipet small drops of the liquid to be tested, keep 1 hour at 38°, pour a saturated aqueous $CuSO_4$ solution over the surface, allow to stand 10 minutes, and rinse with H_2O. The presence of lipase is shown by the appearance of beautiful bluish green spots. These are copper soap. The addition of the starch, which is not indispensable, produces a rather white opaque background against which the spots appear very distinct.

4. Ethyl Butyrate Test.—Into each of two test-tubes introduce 4 c.c. of water, 2 c.c. of ethyl butyrate, $C_3H_7COO.C_2H_5$, and a small amount of litmus powder. To the contents of one tube add 4 c.c. of neutral pancreatic extract and to the contents of the other tube add 4 c.c. of water or of boiled neutral pancreatic extract. Keep the tubes at 40°C. and observe any change which may occur. What is the result and how do you explain it? Write the equation for the reaction which has taken place.

Experiments on Pancreatic Rennin

Prepare four test-tubes as follows:

(a) 5 c.c. of milk + 10 drops of neutral pancreatic extract.

(b) 5 c.c. of milk + 20 drops of neutral pancreatic extract.

[1] Litmus-milk *powder* may be used if desired. To prepare it add 1 part of powdered litmus to 50 parts of dried milk powder. For use in testing, 1 part of powdered litmus-milk may be added to 9 parts of water (Hamilton: *Jour. Bact.*, 6, 43, 1921).

[2] Commercial *pancreatin* may be used in this test if desired.

[3] Carnot and Mauban: *Compt. rend. Soc. Biol.*, 81, 98, 1918; *Chemical Abstracts*, 13, 457, 1919.

(c) 5 c.c. of milk + 10 drops of alkaline pancreatic extract.

(d) 5 c.c. of milk + 20 drops of alkaline pancreatic extract.

Place the tubes at 60°–65°C. for a half hour without shaking. Note the formation of a clot.[1] How does the action of pancreatic rennin compare with the action of the gastric rennin?

Experiments on Erepsin

See page 269.

[1] This reaction will not always succeed, owing to conditions which are not well understood.

CHAPTER XII

INTESTINAL DIGESTION

STRICTLY speaking, all digestive processes which take place in the intestine may be classed under Intestinal Digestion. However, we will consider under Intestinal Digestion only those digestive processes which are brought about by *enzymes which have their origin in the intestine*. The activities of those enzymes which originate in the pancreas we have considered in Chapter X under Pancreatic Digestion.

It has been shown[1] that the reaction of the small intestine may vary from acid to alkaline and is influenced by the state of digestion.

The enzymes of the intestinal juice (*succus entericus*) are of great importance to the animal organism. These enzymes include *erepsin, sucrase, maltase, lactase, nucleases, lipase* and *enterokinase*.

Erepsin is a proteolytic enzyme which has the property of acting upon the proteoses, peptones, and peptides which are formed through the action of trypsin, and further splitting them into *amino-acids*. Erepsin has no power of digesting native proteins. It possesses its greatest activity in an alkaline solution, although it is slightly active in acid solution. An extract of the intestinal erepsin may be prepared by treating the finely divided intestine of a *cat*, *dog*, or *pig* with toluene or chloroform-water and permitting the mixture to stand with occasional shaking for 24–72 hours. Erepsin also occurs in the pancreatic juice and enzymes similar to erepsin occur in various tissues of the organism.

In cases of gastric cancer a peptide-splitting enzyme is claimed to be present in the stomach contents. The glycyl-tryptophane test is sometimes used for its detection. Some investigators claim that the peptide-splitting power of gastric juice in cancer is generally due to the regurgitation of trypsin or erepsin from the intestine or to the presence in the gastric contents of swallowed saliva which possesses peptolytic power. The peptide-splitting power of saliva may be due to a specific enzyme or to the presence of bacteria (see Glycyl-tryptophane Reaction, page 270).

The three invertases *sucrase*, *maltase*, and *lactase* are also important enzymes of the intestinal mucosa. The *sucrase* acts upon sucrose and inverts it with the formation of *invert sugar* (glucose and fructose). Some investigators claim that sucrase is also present in saliva and gastric juice. It probably does not exist normally in either of these

[1] Long and Fenger: *Jour. Am. Chem. Soc.*, 39, 1278, 1917.

digestive juices, however, and if found owes its presence to the excretory processes of certain bacteria. Sucrases may also be obtained from several vegetable sources. For investigational purposes it is ordinarily obtained from yeast (see page 59). It exhibits its greatest activity in the presence of a slight acidity, but if the acidity be increased to any extent the reaction is inhibited.

Lactase is an enzyme which inverts lactose with the consequent formation of glucose and galactose. Its action is entirely analogous, in type, to that of sucrase. It has apparently been proven that lactase occurs in the intestinal mucosa of the young of all animals which suckle their offspring.[1] It may also occur in the intestinal mucosa of certain adult animals if such animals be maintained upon a ration containing more or less lactose. Fischer and Armstrong have demonstrated the reversible action[2] of lactase.

Maltase possesses the power of splitting maltose, the end-product of the digestion of starch, into glucose. It was first discovered in the urine and shortly after this time its presence was noted in the small intestine and the saliva. Corn is sometimes used as the medium for the preparation of the enzyme for experimental purposes. It occurs in corn in a very active state. It was in connection with maltase that the principles of the "reversibility of enzyme action" were first demonstrated.

Enterokinase possesses the power of "activating" trypsinogen (see Chapters II and XI). In other words, trypsinogen as formed by the pancreas has no proteolytic power, but when this inactive trypsinogen reaches the intestine and comes into contact with enterokinase the latter transforms it into active trypsin. Enterokinase is not always present in the intestinal juice since it is secreted only after the pancreatic juice reaches the intestine. It resembles the enzymes in that its activity is destroyed by heat, but differs materially from this class of bodies in that a certain quantity is capable of activating only a definite quantity of trypsinogen. It is, however, generally classified as an enzyme. Enterokinase has been detected in the higher animals, and a kinase possessing similar properties has been shown to be present in bacteria, fungi, impure fibrin, lymph glands, and snake-venom.

The intestinal juice and the epithelium of the intestinal wall contain enzymes capable of hydrolyzing nucleic acids and as these acids are not acted upon by the gastric juice and probably not to any great extent by pure pancreatic juice, the intestine apparently plays the chief rôle in decomposition or digestion of these substances. At least two

[1] Mendel and Mitchell: *American Journal of Physiology*, 20, 81, 1907.
[2] See p. 49.

enzymes take part in this digestion process, one decomposing the nucleic acid with formation of simple nucleotides containing a single radical each of phosphoric acid, carbohydrate and base (see chapter on Nucleic Acids). This enzyme may be called *nucleicacidase*. Another enzyme present in the intestine and intestinal juice decomposes these nucleotides with the liberation of phosphoric acid. This enzyme may be called *nucleotidase* or *phosphonuclease*. The intestinal mucosa also decomposes many other organic phosphorus compounds with liberation of their phosphoric acid.[1] Thus glycero-phosphoric acid and hexose-phosphoric acid as well as phosphoproteins are split in a similar manner, the phosphoric acid they contain being absorbed in the free form.

General Experiments on Intestinal Digestion

Demonstration of Enterokinase.—Trypsinogen may be activated by enterokinase. This activation occurs normally in the intestine. Calcium salts also bring about a similar activation of the trypsinogen.

Procedure.—Prepare an extract of trypsinogen by grinding 10 grams of the fresh, fat-free pancreas of the pig with a little sand. Gradually add 100 c.c. of water during the grinding process. Strain through cheese cloth.

Prepare an extract of enterokinase by grinding 5 grams of fresh, fat-free duodenal mucosa[2] of the pig with a little sand. Gradually add 50 c.c. of water during the grinding process. Strain through cheese cloth.

Prepare the following series of tubes:

(a) 10 c.c. pancreas extract+5 c.c. water.
(b) 10 c.c. pancreas extract+5 c.c. duodenal extract.
(c) 5 c.c. duodenal extract+10 c.c. water.
(d) 10 c.c. pancreas extract +5 c.c. duodenal extract.
(e) 10 c.c. pancreas extract+5 c.c. duodenal extract (boiled).
(f) 10 c.c. pancreas extract+5 c.c. of 4 per cent calcium chloride.

Boil the contents of tube (d) for five minutes and cool to 40°C. Keep all six tubes at 40°C. for 20 minutes.

To each tube add 5 c.c. of 10 per cent sodium carbonate and mix the contents thoroughly and immediately. Introduce into each tube the same quantity (size of a pea) of fresh fibrin. Shake the tubes and place them at 40°C. Observe the tubes frequently for one hour to note digestive changes. Tubes (b) and (f) should show most rapid digestion. Why?

Experiments on Intestinal Nucleases

1. Preparation of Intestinal Extract.—Wash thoroughly 100 grams of pig's intestine and run through a meat chopper several times. Introduce into a 500 c.c. mixing cylinder and add normal salt solution to make 500 c.c. Allow to

[1] Plimmer: *Biochem. J.*, 7, 43, 1913.
[2] The dried mucosa may be substituted if desired.

stand for 6–24 hours at room temperature, shaking occasionally, toluene being added as a preservative. Strain and filter.

2. Demonstration of Intestinal Nucleases.—Prepare a 2 per cent solution of yeast nucleic acid put in solution with the aid of just sufficient NaOH solution to make the resulting mixture neutral to litmus. To each of two large test-tubes add 20 c.c. of the intestinal extract prepared as above. Boil one for one to two minutes. To each tube then add 10 c.c. of the 2 per cent nucleic acid solution. Add 2–3 c.c. each of toluene and chloroform to each mixture. Keep at 38°C. for 24 hours.

Heat the tubes to boiling in a water-bath to coagulate protein. Add 5 c.c. of 5 per cent HCl and allow to stand for one hour. This precipitates any unchanged nucleic acid. Filter and take aliquots of the filtrate (about 20 c.c.). Precipitate the phosphate from each mixture by adding 5 c.c. of magnesia mixture and 5 c.c. of ammonia. Allow to stand over night. A heavy precipitate of magnesium ammonium phosphate should be found in the test experiment indicating that the phosphoric acid of the nucleic acid had been liberated by the nucleotidase of the intestinal extract. The control should show only a slight precipitate.

If desired the phosphorus of the precipitates may be determined quantitatively by dissolving in 2 per cent HNO_3, precipitating as the phosphomolybdate and determining volumetrically according to the procedure given under Total Phosphorus, Chapter XXXI.

Experiments on Erepsin

1. Preparation of Erepsin.—Grind the mucous membrane of the small intestine of a cat, dog, or pig with sand in a mortar. Treat the finely divided membrane with toluene or chloroform-water and permit the mixture to stand, with occasional shaking, for 24–72 hours.[1] Filter the extract thus prepared through cotton and use the filtrate in the following experiment.

2. Demonstration of Erepsin.—To about 5 c.c. of a 1 per cent solution of Witte's peptone in a test-tube add about 1 c.c. of the erepsin extract prepared as described above and make the mixture slightly alkaline (0.1 per cent) with sodium carbonate. Prepare a second tube containing a like amount of peptone solution but boil the erepsin extract before introducing it. Place the two tubes at 38°C. for two to three days. At the end of that period heat the contents of each tube to boiling, filter and try the biuret test on each filtrate. In making these tests care should be taken to use like amounts of filtrate, potassium hydroxide and copper sulphate in each test in order that the drawing of correct conclusions may be facilitated. The contents of the tube which contained the boiled extract should show a deep pink color with the biuret test, due to the peptone still present. On the other hand, the biuret test upon the contents of the tube containing the unboiled extract should be negative or exhibit, at the most, a faint pink or blue color, signifying that the peptone, through the influence of the erepsin, has been transformed, in great part at least, into amino-acids which do not respond to the biuret test.[2]

[1] The enzyme may also be extracted by means of *glycerol* or *alkaline "physiological" salt solution* if desired.

[2] *Strictly speaking*, this erepsin demonstration is not adequate unless a control test is made with *native protein* to show that the extract is *trypsin-free* and digests peptone but not native protein.

3. The Glycyl-tryptophane Reaction.—The dipeptide glycyl-tryptophane[1] may be used in place of the peptone solution for the demonstration of erepsin. It is claimed that a peptide-splitting enzyme (erepsin) is present in the stomach contents of individuals suffering from cancer of the stomach, whereas the stomach contents of normal individuals contain no such enzyme. The glycyl-tryptophane test, therefore, may sometimes furnish a means of aiding in the diagnosis of this disorder. As applied to stomach contents, the test is as follows:[2] Introduce about 10 c.c. of the filtrate from the stomach contents into a test-tube, add a little glycyl-tryptophane and a layer of toluene, and place the tube in an incubator at 38°C. for 24 hours. At the end of this time by means of a pipette transfer 2–3 c.c. of the fluid from beneath the toluene to a test-tube, add a few drops of 3 per cent acetic acid and carefully introduce bromine vapors. Shake the tube and note the production of a red color if tryptophane is present. The tryptophane has, of course, been liberated from the peptide through the action of the peptide-splitting enzyme (erepsin) elaborated by the cancer tissue.

If an excess of bromine is added the color will vanish. If no rose color is noted, add more bromine vapors carefully with shaking until further addition of the vapors causes the production of a yellowish color. This indicates an excess of bromine and constitutes a negative test. Occasionally the rose color indicating a positive test is so transitory as to escape detection unless the test be very carefully performed.

Several fallacies have been pointed out in connection with this test. In the first place the regurgitation of duodenal contents through the pylorus might insure the presence in the stomach of erepsin and trypsin either of which possesses peptide-splitting power. It has also been claimed that saliva contains an enzyme capable of splitting glycyl-tryptophane. Doubt has, however, been cast upon the dipeptide-splitting agent of the saliva by Smithies[3] and by Jacque and Woodyatt,[4] who point to bacteria as the peptolytic agents. In any event saliva contains something which is capable of splitting the glycyltryptophane, thus making the entrance of saliva into the stomach an important source of error, so far as the utility of this test is concerned, as a diagnostic aid. Bacteria may, of course, be removed from the gastric juice by passing the fluid through an effective filter.

Experiments on Invertases[5]

1. Preparation of an Extract of Sucrase.—Treat the finely divided epithelium of the small intestine of a dog, pig, rat, rabbit, or hen with about 3 volumes of a 2 per cent solution of sodium fluoride and permit the mixture to

[1] This dipeptide is sold under the name "Ferment Diagnosticon."
[2] Neubauer and Fischer: *Deutsches Archiv f. klinische Medizin*, 97, 499, 1909.
[3] Smithies: *Arch. Int. Med.*, 10, 521, 1912.
[4] Jacque and Woodyatt: *Arch. Int. Med.*, Dec., 1912, p. 560.
[5] "The Inverting Enzymes of the Alimentary Tract," Mendel and Mitchell: *American Journal of Physiology*, 20, 81, 1907–08.

stand at room temperature for 24 hours. Strain the extract through cloth or absorbent cotton and use the strained material in the following demonstration.

2. Demonstration of Sucrase.—To about 5 c.c. of a 1 per cent solution of sucrose, in a test-tube, add about 1 c.c. of a 2 per cent sodium fluoride intestinal extract, prepared as described above. Prepare a control tube in which the intestinal extract is boiled before being added to the sugar solution. Place the two tubes at 38°C. for two hours.[1] Heat the mixture to boiling to coagulate the protein material, filter, and test the filtrate by Benedict's test (see page 76). The tube containing the boiled extract should give no response to Benedict's test, whereas the tube containing the unboiled extract should reduce the Benedict's solution. This reduction is due to the formation of invert sugar (see page 90) from the sucrose through the action of the enzyme sucrase which is present in the intestinal epithelium.

For preparation and demonstration of Vegetable Sucrase see Chapter II.

3. Preparation of an Extract of Lactase.—Treat the finely divided epithelium of the small intestine of a kitten, puppy, or pig embryo with about 3 volumes of a 2 per cent solution of sodium fluoride and permit the mixture to stand at room temperature for 24 hours. Strain the extract through cloth or absorbent cotton and use the strained material in the following demonstration.

4. Demonstration of Lactase.[2]—To about 5 c.c. of a 1 per cent solution of lactose in a test-tube add about 1 c.c. of a toluene-water or a 2 per cent sodium fluoride extract of the first part of the small intestine[3] of a kitten, puppy, or pig embryo prepared as described above. Prepare a control tube in which the intestinal extract is boiled before being added to the sugar solution. Place the two tubes at 38°C. for 24 hours. At the end of this period add 1 c.c. of the digestion mixture to 5 c.c. of Barfoed's reagent[4] and place the tubes in a boiling water-bath.[5] Examine the tubes at the end of three minutes against a black background in a good light. If no cuprous oxide is visible replace the tubes and repeat the examination at the end of the fourth and fifth minutes. If no reduction is then observed permit the tubes to stand at room temperature for 5–10 minutes and examine again.[6]

It has been determined that disaccharide solutions will not reduce Barfoed's reagent until after they have been heated for 9–10 minutes on a boiling water-bath in contact with the reagent.[7] Therefore in the above test, if the tube containing the unboiled extract exhibits any reduction after being heated as indicated, for a period of five minutes or less, and the control tube containing boiled extract shows no reduction, it may be concluded that lactase was present in the intestinal extract.[8]

[1] If a positive result is not obtained in this time permit the digestion to proceed for a longer period.

[2] Roaf: *Biochemical Journal*, 3, 182, 1908.

[3] Duodenum and first part of jejunum.

[4] To 4.5 grams of neutral crystallized copper acetate in 900 c.c. of water add 0.6 c.c. of glacial acetic acid and make the total volume of the solution 1 liter.

[5] Care should be taken to see that the water in the bath reaches at least to the upper level of the contents of the tubes.

[6] Sometimes the drawing of conclusions is facilitated by pouring the mixture from the tube and examining the bottom of the tube for adherent cuprous oxide.

[7] The heating for 9-10 minutes is sufficient to transform the disaccharide into monosaccharide.

[8] The reduction would, of course, be due to the action of the *glucose* and *galactose* which had been formed from the lactose through the action of the enzyme *lactase*.

5. Preparation of an Extract of Maltase.—Treat the finely divided epithelium of the small intestine of a cat, kitten, or pig (embryo or adult) with about 3 volumes of a 2 per cent solution of sodium fluoride and permit the mixture to stand at room temperature for 24 hours. Strain the extract through cloth and use the strained material in the following demonstration.

6. Demonstration of Maltase.—Proceed exactly as indicated in the demonstration of lactase, p. 271, except that a 1 per cent solution of maltose is substituted for the lactose solution. The extract used may be prepared from the upper part of the intestine of a cat, kitten, or pig (embryo or adult). In the case of lactase, as indicated, the intestine used should be that of a kitten, puppy, or pig (embryo).

CHAPTER XIII

BILE

The bile is secreted continuously by the liver and passes into the intestine through the common bile duct which opens near the pylorus. This process is continuous even during prolonged fasting,[1] provided there is no obstruction. It flows more rapidly, however, when food is taken and the length of time elapsing between the ingestion of the food and the secretion of the bile as well as the qualitative and quantitative characteristics of the secretion depending upon the nature of the food ingested. Fats, the extractives of meat and the protein end-products of gastric digestion (proteoses and peptones), cause a copious secretion of bile, whereas such substances as water, acids and boiled starch paste fail to do so. In general a rich protein diet is supposed to increase the amount of bile secreted, whereas a carbohydrate diet would cause a much less decided increase and might even tend to decrease the amount. It has been demonstrated by Bayliss and Starling that the secretion of bile is under the control of the same mechanism that regulates the flow of pancreatic juice (see page 255). In other words, the hydrochloric acid of the chyme, as it enters the duodenum transforms prosecretin into secretin and this in turn enters the circulation, is carried to the liver, and stimulates the bile-forming mechanism to increased activity.

We may look upon the bile as an *excretion* as well as a *secretion*. In the fulfillment of its excretory function it passes such bodies as lecithin, metallic substances, cholesterol, and the decomposition products of hemoglobin into the intestine and in this way aids in removing them from the organism. The bile assists materially in the absorption of fats from the intestine by its solvent action on the fatty acids formed by the action of the pancreatic juice.

The bile is a ropy, viscid substance which is usually alkaline in reaction to litmus (pH 7.8), and ordinarily possesses a decidedly bitter taste. It varies in color in the different animals, the principal variations being yellow, brown and green. Fresh human bile from the living organism ordinarily has a green or golden-yellow color. Post-mortem bile is variable in color. It is very difficult to determine accurately the amount of normal bile secreted during any given period. For an adult

[1] Carlson: *Jour. Am. Med. Ass'n*, 85, 1468, 1925.

man it has been variously estimated at from 500 c.c. to 1100 c.c. for 24 hours. The specific gravity of the bile varies between 1.010 and 1.040 and the freezing-point is about −0.56°C. As secreted by the liver, the bile is a clear, limpid fluid which contains a relatively low content of solid matter. Such bile would have a specific gravity of approximately 1.010. After it reaches the gall-bladder, however, it becomes mixed with mucous material from the walls of the gall-bladder, and this process coupled with the continuous absorption of water from the bile has a tendency to concentrate the secretion. Therefore the bile as we find it in the gall-bladder ordinarily possesses a higher specific gravity than that of the freshly secreted fluid. The specific gravity under these conditions may run as high as 1.040.

The principal constituents of the bile are the *salts of the bile acids*, *bile pigments*, *neutral fats*, *lecithin*, *phosphatides*, *nucleoprotein*, *mucin*, and *cholesterol*, besides the salts of *iron*, *copper*, *calcium*, and *magnesium*. *Zinc* has also frequently been found in traces.

The *quantitative* composition of bile varies according to the source of the bile, *i.e.*, whether the bile for analysis is obtained from the gall-

QUANTITATIVE COMPOSITION OF BILE[1]

(Parts per 1000)

Constituent	Bladder bile, Hammarsten[2]	Biliary fistula, Rosenbloom[3]
Water	829.7	970.2
Solids	170.3	39.8
Bile salts	97.0	10.1
Mucin and pigments	41.9	4.86
Cholesterol	9.9	2.61[5]
Fat	1.9	6.85
Soaps	11.2[4]	2.6
Lecithin	2.2	6.42
Inorganic matter	5.1	9.2
Fatty acids	Included under "soaps"	1.2

[1] For other analyses see Czylharz, Fuchs and v. Fürth: *Bioch. Zeit.*, 49, 120, 1913.
[2] Hammarsten: *Pincussohn's Med.-Chem. Lab. Hilfsbuch*, Leipzig, 1912, p. 388.
[3] Rosenbloom: *Jour. Biol. Chem.*, 14, 241, 1913.
[4] Includes fatty acids.
[5] Includes cholesterol esters.

bladder or by means of a fistula before it reaches the gall-bladder. The variation in the composition of these two types of bile is shown in the preceding selected analyses.

The bile acids,[1] which are elaborated exclusively by the hepatic cells, may be divided into two groups, the *glycocholic acid* group and the *taurocholic acid* group. In human bile glycocholic acid predominates, while taurocholic acid is the more abundant in the bile of carnivora. The bile acids are conjugate *amino-acids*, the glycocholic acid yielding *glycocoll*,

$$\begin{array}{l} CH_2 \cdot NH_2 \\ | \\ COOH, \end{array}$$

and *cholic acid* upon decomposition, whereas taurocholic acid gives rise to *taurine*,

$$\begin{array}{l} CH_2 \cdot NH_2 \\ | \\ CH_2 \cdot SO_2 \cdot OH, \end{array}$$

and *cholic acid* under like conditions. Glycocholic acid contains some nitrogen but no sulphur, whereas taurocholic acid contains both these elements. The sulphur of the taurocholic acid is present in the taurine (amino-ethyl-sulphonic-acid), of which it is a characteristic constituent. There are several varieties of cholic acid and therefore we have several forms of glycocholic and taurocholic acids, the variation in constitution depending upon the nature of the cholic acid which enters into the combination. The bile acids are present in the bile as salts of one of the alkalis, generally sodium. The sodium glycocholate and sodium taurocholate may be isolated in crystalline form, either as balls or rosettes of fine needles or in the form of prisms having ordinarily four or six sides (Fig. 79, page 276). The salts of the bile acids are dextro-rotatory. Among other properties these salts have the power of holding the cholesterol and lecithin of the bile in solution. They also have the power of reducing surface tension, and thus assist in the emulsification of fats.

It has been shown[2] that a functionally defective liver (Eck fistula) produces less than one-half the normal amount of bile acid. This is direct evidence that the bile acids are formed essentially by liver cell activity.

Hammarsten has demonstrated a third group of bile acids in the bile of the shark. This same group very probably occurs in certain other animals also. These acids are very rich in sulphur and resemble ethereal sulphuric acids inasmuch as upon treatment with boiling hydrochloric acid they yield sulphuric acid.

[1] Smyth and Whipple: *Jour. Biol. Chem.*, 59, 623, 1924.
[2] Foster, Hooper, and Whipple: *Jour. Biol. Chem.*, 38, 393, 1920.

The bile pigments are important and interesting biliary constituents. The following have been isolated: *bilirubin*, *biliverdin*, *bilifuscin*, *biliprasin*, *bilihumin*, *bilicyanin*, *choleprasin*, and *choletelin*. Of these, bilirubin and biliverdin are the most important and predominate in normal bile. The colors possessed by the different varieties of normal

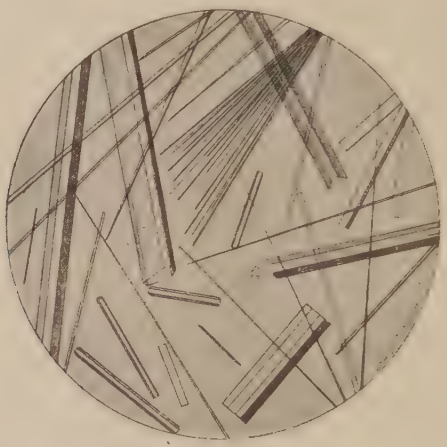


FIG. 79.—BILE SALTS.

bile are due almost entirely to these two pigments, the biliverdin being the predominant pigment in greenish bile and the bilirubin being the principal pigment in lighter colored bile. The pigments, other than the two just mentioned, have been found almost exclusively in biliary calculi or in altered bile obtained at post-mortem examinations.

FIG. 80.—BILIRUBIN (HEMATOIDIN). (*Ogden.*)

Bilirubin, which is perhaps the most important of the bile pigments, is apparently derived from the blood pigment, the iron freed in the process being held in the liver. Bilirubin has the same percentage composition as hematoporphyrin, which may be produced from hematin. It is not a specific product of the liver cells, but is formed in certain other parts of the body as well, *e.g.* spleen and bone marrow. This has

been shown by Mann and associates[1] who found that bilirubin was still formed after removal of the liver. This finding is of importance in considerations of jaundice. It has been suggested that the bilirubin formed in the liver is not identical in character with that formed in other parts of the body.[2] Bilirubin may be isolated in the form of a reddish-yellow powder or may be obtained in part, in the form of reddish-yellow rhombic plates (Fig. 80) upon the spontaneous evaporation of its chloroform solution. The crystalline form of bilirubin is practically the same as that of hematoidin. It is easily soluble in chloroform, somewhat less soluble in alcohol and only slightly soluble in ether and benzene. Bilirubin has the power of combining with certain metals, particularly calcium, to form combinations which are no longer soluble in the solvents of the unaltered pigment. Upon long standing in contact with the air, the reddish-yellow bilirubin is oxidized with the formation of the green biliverdin. Bilirubin occurs in animal fluids as soluble bilirubin-alkali. The accumulation of bilirubin in the blood may serve as an index of biliary obstruction or blood destruction.

Solutions of bilirubin exhibit no absorption bands. If an ammoniacal solution of bilirubin-alkali in water is treated with a solution of zinc chloride, however, it shows bands similar to those of bilicyanin (Absorption Spectra, Plate II), the two bands between C and D being rather well defined.

Biliverdin is particularly abundant in the bile of herbivora. It is soluble in alcohol and glacial acetic acid and insoluble in water, chloroform, and ether. Biliverdin is formed from bilirubin upon oxidation. It is an amorphous substance, and in this differs from bilirubin which may be at least partly crystallized under proper conditions. Biliverdin may be obtained in the form of a green powder. In common with bilirubin, it may be converted into hydrobilirubin by nascent hydrogen.

The neutral solution of bilicyanin or cholecyanin is bluish green or steel blue and possesses a blue fluorescence, the alkaline solution is green with no appreciable fluorescence, and the strongly acid solution is violet blue. The alkaline solution exhibits three absorption bands, the first a dark, well-defined band between C and D, somewhat nearer C; the second a less sharply defined band extending across D and the third a rather faint band between E and F, near E (Absorption Spectra, Plate II). The strongly acid solution exhibits two absorption bands, both lying between C and E and separated by a narrow space near D. A third band, exceedingly faint, may ordinarily be seen between b and F.

[1] Mann: *Jour. Am. Med. Ass'n.*, 85, 1472, 1925.
Mann, Bollman and Magath: *Am. Jour. Physiol.*, 69, 393, 1924.
Mann, Sheard, Bollman and Baldes: *Ibid.*, 74, 497, 1925; 77, 219, 1926
[2] Van den Bergh: *Presse Medicale*, 29, 441, 1921.

Bile pigments are converted into *urobilinogen* (urobilin) in the intestine. This is absorbed, carried to the liver and *reconverted into bile pigment.* In diseases of the liver the liver cell loses the capacity to convert the urobilinogen and this is then excreted in the urine. The presence of urobilinogen in urine, therefore, may be considered as an index of *functional liver incapacity.*[1] (See Chapter XXXI.)

Biliary calculi, otherwise designated as *biliary concretions* or *gall stones*, are frequently formed in the gall-bladder. These deposits may be divided into five classes, *cholesterol calculi*, *cholesterol-calcium calculi*, *cholesterol-calcium–pigment calculi*, *calcium-pigment calculi*, and calculi made up almost entirely of *inorganic material.* This last class of calculus is formed principally of the carbonate and phosphate of calcium and is rarely found in man although quite common to cattle. The calcium-pigment calculus is also found in cattle, but is more common to man than the inorganic calculus. This calcium-pigment calculus ordinarily consists principally of bilirubin in combination with calcium; biliverdin is sometimes found in small amount. The cholesterol calculus is the one found most frequently in man. These may be formed almost entirely of cholesterol, in which event the color of the calculus is very light, or they may contain more or less pigment and inorganic matter mixed with the cholesterol, which tend to give calculi of various colors. The cholesterol content of calculi may be derived from the walls of the gall bladder or biliary tract or from the bile itself. The blood in certain cases of cholelithiasis may also have a high cholesterol content.[2]

For discussion of cholesterol see Chapter XXV.

Experiments on Bile

1. Reaction.—Test the reaction of fresh ox bile to litmus, phenolphthalein and Congo red.

2. Nucleoprotein.—Acidify a small amount of bile with dilute acetic acid. A precipitate of nucleoprotein forms. Bile acids will also precipitate here under proper conditions of acidity.

3. Inorganic Constituents.—Test for chlorides, sulphates, and phosphates (see page 109).

4. Tests for Bile Pigments.—Practically all of these tests for bile pigments are based on the oxidation of the pigment, by a variety of reagents, with the formation of a series of colored derivatives, *e.g.*, biliverdin (green), bilicyanin (blue), choletelin (yellow). However the formation of a yellow foam on shaking the solution is a sensitive test for bile.

[1] Rowntree, Hurwitz and Bloomfield: *Johns Hopkins Hospital Bulletin*, Nov., 1913. Wallace and Diamond: *Arch. Int. Med.*, 35, 698, 1925.

[2] For discussion of gall stone formation see Wells: "Chemical Pathology," 5th ed., p. 506, 1925.

(a) Gmelin's Test.—To about 5 c.c. of concentrated nitric acid in a test-tube add 2–3 c.c. of diluted bile carefully so that the two fluids do not mix. At the point of contact note the various colored rings, green, blue, violet, red and reddish yellow. Repeat this test with different dilutions of bile and observe its delicacy.

(b) Rosenbach's Modification of Gmelin's Test.—Filter 5 c.c. of diluted bile through a small filter paper. Introduce a drop of concentrated nitric acid into the cone of the paper and note the succession of colors as given in Gmelin's test.

(c) Huppert-Cole Test.[1]—Boil about 15 c.c. of the fluid in a test tube. Add two drops of a saturated solution of magnesium sulphate, then add a 10 per cent solution of barium chloride, drop by drop, boiling after each addition. Continue to add the barium chloride until no further precipitate is obtained.[2] Allow the tube to stand for a minute. Pour off the supernatant fluid as cleanly as possible or use a centrifuge. To the precipitate add 3 to 5 c.c. of 97 per cent alcohol, two drops of strong sulphuric acid, and two drops of a 5 per cent aqueous solution of potassium chlorate. Boil for half a minute and allow the barium sulphate to settle. The presence of bile pigments is indicated by the alcoholic solution being colored a greenish blue.

NOTES.—To render the test more delicate, pour off the alcoholic solution from the barium sulphate into a dry tube. Add about one-third its volume of chloroform and mix. To the solution add about an equal volume of water, place the thumb on the tube, invert once or twice and allow the chloroform to separate. It contains the bluish pigment in solution.

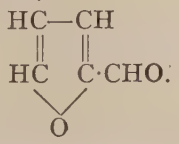
The bile pigment is adsorbed on to the barium sulphate precipitate, but passes into solution again in acid alcohol. The chlorate acts as a very weak oxidizing reagent, converting bilirubin and biliverdin to the characteristic blue compound.

The author claims that it is a very much more delicate test than Gmelin's Test.

5. Test for Bile Acids.—(a) Sucrose-H_2SO_4 Test (Pettenkofer).—To 5 c.c. of diluted bile in a test-tube add 5 drops of a 5 per cent solution of sucrose. Now run about 2–3 c.c. of concentrated sulphuric acid carefully down the side of the tube and note the red ring at the point of contact. Upon slightly agitating the contents of the tube the whole solution gradually assumes a reddish color. As the tube becomes warm, it should be cooled in running water in order that the temperature of the solution may not rise above 70°C.

It is claimed that this test is not satisfactory in the presence of protein and chromogenic substances which yield interfering colors with sulphuric acid.

(b) Furfural-H_2SO_4 Test.—Mylius's Modification of Pettenkofer's Test.—To approximately 5 c.c. of diluted bile in a test-tube add 3 drops of a very dilute (1 : 1000) aqueous solution of furfural,



Now run about 2–3 c.c. of concentrated sulphuric acid carefully down the side of the tube and note the red ring as above. In this case, also, upon shaking the

[1] Cole's "Practical Physiological Chemistry." 7th edition, p. 323, 1926.

[2] A few drops of each of 10 per cent calcium chloride and potassium hydroxide solutions may also be used to carry down the bile pigment.

tube the whole solution is colored red. Keep the temperature of the solution below 70°C. as before.

(c) Foam Test (v. Udránsky).—To 5 c.c. of diluted bile in a test-tube add 3–4 drops of a very dilute (1 : 1000) aqueous solution of furfural. Place the thumb over the top of the tube and shake the tube until a thick foam is formed. By means of a small pipette add 2–3 drops of concentrated sulphuric acid to the foam and note the dark pink coloration produced.

(d) Surface Tension Test (Hay).—This test is based upon the principle that bile acids have the property of reducing the surface tension of fluids in which they are contained. The test is performed as follows: Cool about 10 c.c. of diluted bile in a test-tube to 17°C. or lower and sprinkle a little finely pulverized sulphur upon the surface of the fluid. The presence of bile acids is indicated if the sulphur sinks to the bottom of the liquid, the rapidity with which the sulphur sinks depending upon the quantity of bile acids present in the mixture. The test is said to detect bile acids when they are present in the ratio of 1 : 120,000.

(e) Peptone Test (Oliver).—To 5 c.c. of diluted bile add 2–3 drops of acetic acid, filtering if necessary. Add an equal volume of a 1 per cent solution of Witte's peptone to the acid solution. A precipitate is produced which is insoluble in excess of acetic acid. This precipitate is a compound of protein and bile acids.

6. Crystallization of Bile Salts.—To 25 c.c. of undiluted bile in an evaporating dish add enough animal charcoal to form a paste and evaporate to dryness on a water-bath. Remove the residue, grind it in a mortar, and transfer it to a small flask. Add about 50 c.c. of absolute alcohol and boil on a water-bath for 20 minutes. Filter, and add ether to the filtrate until there is a slight permanent cloudiness. Cover the vessel and set it aside until crystallization is complete. Examine the crystals under the microscope and compare them with those shown in Fig. 79, page 276. Try one of the tests for bile acids upon some of the crystals.

7. Analysis of Biliary Calculi.—Grind the calculus in a mortar with 10 c.c. of ether. Filter.

Filtrate I.

Add an equal volume of 95 per cent alcohol[1] to the ether extract, allow the mixture to evaporate and examine for *cholesterol* crystals (Fig. 81, page 281). (For further tests see Experiment 8, below.)

Residue I.
(On paper and in mortar.)

Treat with dilute hydrochloric acid and filter.

Filtrate II.

Test for *calcium*, *phosphates*, and *iron*. Evaporate remainder of filtrate to dryness in porcelain crucible and ignite. Dissolve residue in dilute hydrochloric acid and make alkaline with ammonium hydroxide. Blue color indicates *copper*.

Residue II.
(On paper and in mortar.)

Wash with a little water. Dry the filter paper.

Treat with 5 c.c. chloroform and filter.

Filtrate III.
Bilirubin.
(Apply test for bile pigments.)

Residue III.
(On paper and in mortar.)

Treat with 5 c.c. of *hot* alcohol.

Biliverdin.

[1] The alcohol is added because of the fact that it is often found that crystallization from pure ether does not yield typical cholesterol crystals.

8. Tests for Cholesterol.[1]

(a) Microscopical Examination.—Examine the crystals under the microscope and compare them with those shown in Fig. 81, below.

(b) Sulphuric Acid Test (Salkowski).—Dissolve a few crystals of cholesterol in a little chloroform and add an equal volume of concentrated sulphuric acid. A play of colors from bluish-red to cherry-red and purple is noted in the chloroform while the acid assumes a marked green fluorescence.

(c) Acetic Anhydride-H_2SO_4 Test (Liebermann-Burchard).—Dissolve a few crystals of cholesterol in 2 c.c. of chloroform in a dry test-tube. Now add 10 drops of acetic anhydride and 1–3 drops of concentrated sulphuric acid. The solution becomes red, then blue, and finally bluish-green in color. This reaction is used in the quantitative determination of cholesterol (see Chapter XVIII).

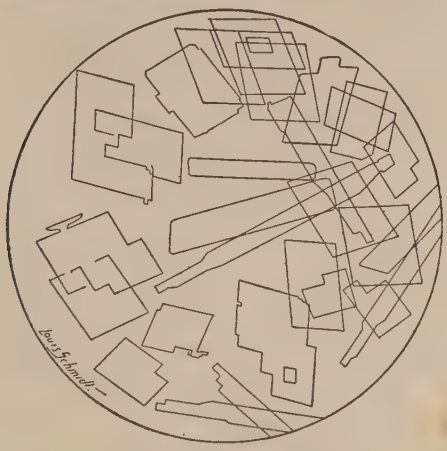


FIG. 81.—CHOLESTEROL.

9. Preparation of Taurine.—To 300 c.c. of bile in a casserole add 100 c.c. of hydrochloric acid and heat until a sticky mass (dyslysin) is formed. This point may be determined by drawing out a thread-like portion of the mass by means of a glass rod, and if it solidifies immediately and assumes a brittle character we may conclude that all the taurocholic and glycocholic acid has been decomposed. Decant the solution and concentrate it to a small volume on the water-bath. Filter the hot solution to remove sodium chloride and other substances which may have separated, and evaporate the filtrate to dryness. Dissolve the residue in 5 per cent hydrochloric acid and precipitate with 10 volumes of 95 per cent alcohol. Filter off the taurine and recrystallize it from hot water. (Save the alcoholic filtrate for the preparation of glycocoll, p. 282.) Make the following tests upon the taurine crystals.

(*a*) Examine them under the microscope and compare with Fig. 82.

(*b*) Heat a crystal upon platinum foil. The taurine at first melts, then turns brown, and finally carbonizes as the temperature is raised. Note the suffocating odor. What is it?

(*c*) Test the solubility of the crystals in water and in alcohol.

(*d*) Grind up a crystal with four times its volume of dry sodium carbonate and

[1] For a quantitative method for the determination of cholesterol in bile see McMaster: *Jour. Exp. Med.*, 40, 25, 1924.

fuse on platinum foil. Cool the residue, transfer it to a test-tube, and dissolve it in water. Add a little dilute sulphuric acid and note the odor of hydrogen sulphide. Hold a piece of filter paper, moistened with a *small* amount of lead acetate, over the opening of the test-tube and observe the formation of lead sulphide.

FIG. 82.—TAURINE.

10. Preparation of Glycocoll.—Concentrate the alcoholic filtrate from the last experiment (9) until no more alcohol remains. The glycocoll is present here in the form of an hydrochloride and may be liberated from this combination by the addi-

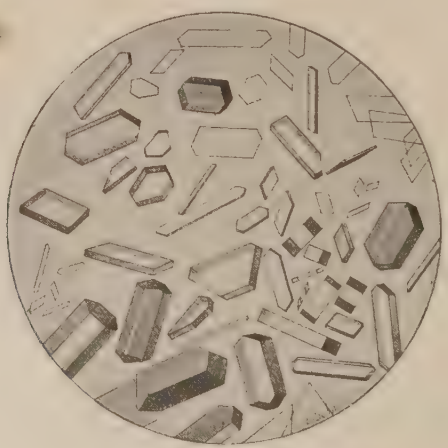


FIG. 83.—GLYCOCOLL. (See also Fig. 135.)

tion of freshly precipitated lead hydroxide or by lead hydroxide solution. Remove the lead by hydrogen sulphide. Filter and decolorize the filtrate by animal charcoal. Filter again, concentrate the filtrate, and set it aside for crystallization. Glycocoll separates as colorless crystals (Fig. 83).

CHAPTER XIV

ABSORPTION

Absorption[1] is primarily a function of the small intestine. Very little absorption takes place from the stomach and the process is nearly complete before the colon is reached. The small intestine is particularly adapted to carry on this process by virtue of its great length (about 27 feet in man) and by the structure of its mucous membrane. The surface of the latter is greatly increased by the presence of the finger-like processes, the villi. They possess a rich blood supply, lymph spaces called lacteals, and muscle fibres whose contractions mechanically assist absorption. The mucous membrane through which substances must pass to enter the blood or lymph is extremely thin but it must not be forgotten that it consists of living cells.

Substances in solution tend to distribute themselves uniformly throughout the border lines of the solvent and this tendency which we measure as osmotic pressure is a powerful force. Undoubtedly osmosis may play an important role in absorption of certain substances such as sugars which may be found in the intestine in far higher concentrations than in the blood. There is much evidence, however, that the process is rarely one of simple diffusion.

The facts with regard to the absorption of ionic substances (salts, amino acids, etc.) have been made much more intelligible by the development of the Donnan theory of membrane equilibria. This explains how it is possible for ionic diffusion to be more or less selective and how certain ions may pass into the blood although present there in higher concentration than in the intestinal lumen.

Even with this extension, however, it is not yet possible to explain many phenomena of absorption on a purely physical basis. Not only do the regulatory powers of the cell and its changing structure influence the course of diffusion processes but it seems probable that certain substances in their passage enter into chemical combination with constituents of the protoplasm thus making possible different types of selective transportation. Furthermore white blood cells actually migrate back and forth between the lymph and the lumen of the intestine. There is evidence that certain of these may actually engulf particles of iron

[1] Goldschmidt: On the Mechanism of Absorption from the Intestine. *Physiol. Reviews*, 1, 421, 1921.

compounds. One of their functions may thus be to assist by phagocytosis in the absorption of difficultly soluble substances. Their number is hardly adequate to their playing a large role in the absorption of the major foodstuffs although fat globules are found in them. However, their accumulation in the intestinal mucosa during digestion would indicate that their function is an important one.

The intestinal mucous membrane further possesses the power of excreting certain substances into the gut. Sugar injected into the blood stream appears in part in the duodenum and calcium salts are eliminated by the mucosa of the large intestine. The glands of the mucosa secrete a digestive fluid, the intestinal juice and the cells themselves contain enzymes such as erepsin, invertases and phosphatases which help to prevent the passage of incompletely decomposed proteins, carbohydrates and organic phosphates into the blood.

Because of the many factors involved, the study of intestinal absorption offers certain difficulties. Investigation of the processes concerned helps, however, to throw light on permeability and other fundamental properties of protoplasm. It is further of the greatest practical importance to understand the factors governing the absorption of the many essential foodstuffs, and to determine the conditions under which there is a loss of the great protective power of the intestinal mucosa to detoxicate or prevent the absorption of toxic substances, which are ingested or produced during the course of digestion or putrefaction and lead to symptoms of chronic or acute disease.

Carbohydrates are absorbed almost entirely as the simple sugars, glucose, fructose and galactose. Of the disaccharides, lactose is least readily hydrolyzed in the intestine and some of it occasionally reaches the blood as such. In these cases it is eliminated unchanged in the urine. Sucrose entering the blood is also excreted as such. When strong sugar solutions enter the bowel they are diluted by the intestinal secretion until the concentration is reduced to a point at which absorption may effectively proceed. Lactose because of its slow digestion and absorption reaches a much lower level in the bowel than other sugars and is thus far more effective in reducing putrefaction.

The simple sugars entering the blood are quite rapidly removed therefrom and stored up in the liver and other tissues as glycogen. After the ingestion of 100 grams of glucose a maximum blood sugar value of about 0.15 per cent is reached in less than an hour, the normal figure of about 0.1 per cent being restored by the end of two hours.

Protein is absorbed in the form of individual amino acids. An increase in the amino acid content of the plasma and more especially of the red corpuscles is noted after a meal. The amount in the red cells

may attain a maximum of 20–25 mg. (expressed as amino nitrogen) in about five hours after protein ingestion. The amino acids are removed quite rapidly from the blood by the tissues which build them up into protoplasm or oxidize them, with the production of urea, water and carbon dioxide. The first step in the oxidation process is deaminization (the splitting off of ammonia). The liver is particularly concerned in this process. Some deaminization may occur in the intestinal wall. The blood of the portal circulation is high in ammonia but much of this arises from intestinal putrefaction in the colon.

That incompletely decomposed protein may sometimes be absorbed is indicated by the production in certain individuals of anaphylactic symptoms (cutaneous eruptions, asthma, etc.) following the ingestion of particular types of protein. Such reactions are not produced by completely decomposed proteins.

Saponification is essential for the absorption of fats.[1] Paraffin oils incapable of hydrolysis are not absorbed. The glycerol formed in saponification is very soluble in water and is readily absorbed unchanged. The higher fatty acids are however but slightly soluble in water. The sodium and potassium soaps formed by reaction with the alkaline pancreatic juice are much more soluble but the solution formed is a colloidal solution. Such solutions possess a very low osmotic pressure and diffusion cannot account for their rapid absorption. That bile plays an important role in fat absorption is shown by the appearance of large amounts of fat and fatty acid in the stools when the bile ducts are occluded. Apparently the fatty acids combine with the bile salts and are absorbed with them into the epithelial cells. In these cells the fatty acids are again combined with glycerol to form fats and pass into the lymph as such. The character of the fat is somewhat modified in the process of absorption so as to more nearly resemble the body fat of the animal. It is stored (more especially in the adipose tissues) or burned. The increase in lecithin of the red cells during fat absorption indicates that this phosphatide represents an intermediate compound in fat metabolism. The greater part of the fat enters the lacteals and then the lymphatics forming an emulsion (the chyle) which pours into the jugular vein through the thoracic duct. An increase in the fat content of the blood and lymph following a meal ("fat-tide") is readily demonstrated.

The selective nature of absorption applies even to inorganic salts. Thus sulphates are much less readily absorbed than chlorides and tend to withdraw water from the blood. For this and other reasons the sulphates have a cathartic effect. The absorption of calcium and phos-

[1] Bloor: *Physiol. Reviews*, 2, 92, 1922.

phorus is of especial interest because of its relation to the development of rickets and because its absorption can be so profoundly effected by minute amounts of antirachitic substances and by ultra violet radiation. The acidity of the intestinal contents is also of importance for the absorption of the relatively insoluble salts of calcium. Calcium, magnesium, phosphorus and iron are excreted to a considerable extent by the intestinal mucosa. Hence a study of their absorption at different levels of the intestinal tract is required for an understanding of the factors involved. The absorptive power of the colon for food substances is relatively low.

Much of our information on absorption has been obtained from the study of isolated intestinal loops which retain their nerve and blood supply. Solutions can be injected into such loops and the contents removed at any time for analysis. The relative rates of absorption of various substances can thus be determined under controlled conditions.

Histological examination of the mucosa of animals killed after a meal has shown the presence of fat globules in the cells.

By establishing fistulas of the intestine the course of absorption in different parts of the tract has been studied.

Analysis of the blood and lymph give information of great importance relative to the nature of the products entering the blood stream. Urine analyses show the rapidity with which soluble inorganic salts are absorbed. Transfusion experiments on the intestine are unsuccessful because a normal mucous membrane cannot be maintained. Studies on absorption from the stomach may be made with a stomach tube and a similar procedure may give some information as to absorption from the colon. Fecal analyses show the completeness of digestion and absorption of various substances present in the food. Cori[1] kills animals a short time after the ingestion of sugar solution, washes out the whole tract and determines how much of the sugar has been absorbed (see p. 287).

Animals may also be fed diets containing a known amount of iron oxide. It has been shown that the iron is not absorbed appreciably. The ratio of any absorbable substance to iron will naturally decrease as absorption proceeds and the percentage absorbed at any level of the intestine can be determined by analysis for iron and for the substance concerned (see p. 288). The course of digestive processes in the stomach and intestines can also be followed as well as the ultimate utilization of the food.

[1] Cori, *Jour. Biol. Chem.*, 66, 691, 1925.

Experiments on Absorption

1. Study of the Absorption of Glucose from the Human Stomach. Strausz Modification of Method of Sahli.[1] Principle.—Potassium iodide is added to the glucose test solution. Potassium iodide appears to be absorbed very slowly from the stomach and therefore its estimation in the gastric contents serves as an index of the absorption of other substances as well as of the motility and secretion of the stomach. Other substances such as urea, or iron salts[2] may also be used.

Procedure.—Pass a retention stomach tube (see p. 225). Remove residuum. Give the subject a mixture of 17.6 gm. glucose with 11 c.c. of 5 per cent potassium iodide solution and weak tea to make 330 c.c. Reserve 30 c.c. for analysis. Designate this solution as (O). After 20 minutes empty the stomach by aspiration. Measure the volume of this mixture (E). Inject 50 c.c. of water. Aspirate and reinject several times to wash the stomach. Again empty. Measure the volume of the lavage (L). Determine glucose in E by Benedict's method Chapter XXXI). Determine iodine in E by first fermenting with yeast to remove glucose and then filtering. Add to 10 c.c. of the filtrate in a separatory funnel, a few c.c. of chloroform and 3 drops of reagent (distil 2 gm. starch and 8.5 c.c. of conc. HNO_3 so that vapors pass into 10 c.c. conc. H_2SO_4 until yellow vapors no longer rise). Shake. Draw off chloroform. Repeat the extraction. Wash the combined extract with water and then titrate with 0.1 N thiosulphate.

Calculation.—One c.c. 0.1 N thiosulphate equals 16.6 mg. KI. The ratios of glucose to iodide in O and in E are calculated. If the ratio for O is 30 and for E is 27 then 90 per cent of the glucose remains and 10 per cent has been absorbed. From the combined iodide of E and L the percentage of iodide which has left the stomach is estimated. This is a measure of motility. From the percentage of iodide in O and E an estimate of the degree of dilution and amount of secretion by the stomach can be made. E should also be titrated for free hydrochloric acid (p. 231).

Interpretation.—Values of 75 per cent for motility, 100 c.c. for secretion, and 10 per cent for absorption have been reported for normal individuals.

2. Determination of Rapidity of Absorption of Sugars from the Entire Gastrointestinal Tract.—Method of Cori.[3] Principle.—Animals are given sugar solutions by a stomach tube. After a suitable time has elasped the animals are killed, the entire gastrointestinal tract removed and total sugar remaining unabsorbed determined.

Procedure.—Rats two to three months old and weighing from 120 to 180 grams are weighed. They are then placed in small wire screen cages with screen bottoms so that there is no access to feces. For 48 hours they are given water but no food. The rats are again weighed and are then fed the solutions to be tested usually 1.25 to 2.5 c.c. of 25 to 80 per cent sugar solutions. These are introduced by means of stomach tubes consisting of Nos. 4 to 5 urethral catheters softened by plunging for a moment into boiling water. A small mouthpiece is used and the catheter marked to indicate depth to which it should be introduced. A syringe of the Record type with needle to connect with catheter is used to inject the fluid. If diarrhea is caused the experiment is discarded.

[1] Anciaes: *Archiv. f. Verdauungskrank.*, 32, 343, 1924.
[2] Bergeim: *Arch. Int. Med.*, 37, 110, 1926.
[3] Cori: *J. Biol. Chem.*, 66, 691, 1925.

An animal is killed at each hourly interval. The entire gastrointestinal tract is removed, slit open and washed thoroughly with water making up to a volume of nearly 500 c.c. A small amount of dialyzed iron is added and then a little sodium sulphate to precipitate it, along with interfering substances. The sugar is determined by the Benedict method or some other method. Blood sugar may also be determined by the Hagedorn-Jensen method or other method. Sugar may also be determined in the urine. By other experiments it can be shown by analyzing the gastric contents for sugar that a dilution of the sugar solution takes place there. The amount of sugar absorbed per 100 gm. of body weight of the animals is calculated. An absorption curve may also be plotted from the results obtained with similar animals at 1, 2, 3, 4, etc. hours. Cori finds that the rate of absorption of hexoses is independent of the concentration and the rates of absorption of some monosaccharides are in the following order: galactose > glucose > fructose > mannose > xylose > arabinose. No glucose appeared in the urine but about 50 per cent of the galactose was excreted by this channel.

The tolerance of animals for sugars injected intravenously may also be determined and an idea obtained as to the rapidity with which sugars are absorbed when injected intraperitoneally as compared with absorption from the intestine. Cori finds the tolerance for glucose given intravenously to be from 2.2 to 2.5 gm. per kilo of body weight per hour.

If a soluble ferric salt such as ferric ammonium citrate be added to the sugar solutions it is possible to determine in what parts of the tract digestion and absorption of carbohydrates, etc., more particularly occur (see experiments below).

3. Influence of Carbohydrates on the Utilization of Calcium and Phosphorus. Method of Bergeim.[1] Principle.—To a standard diet is added a definite proportion of iron oxide and of the carbohydrate whose effect it is desired to study. The ratios of calcium and phosphorus to iron are determined for foods and feces and the percentage absorption calculated. The accurate separation of feces of the experimental period is not necessary.

Procedure.—Feed two or more albino rats (about 60 gm. in weight) on a phosphorus-low and calcium-high diet (whole yellow corn 77, wheat gluten 20, calcium carbonate 3 and C.P. ferric oxide 0.2) for about three weeks. Put in separate cages with screen bottoms. Collect feces for a five day period. Then add 35 per cent of glucose to the diet of one animal and an equal amount of pure lactose to the diet of the other. After two days on these diets collect feces for a period of four days. Then exchange diets so that the first animal gets lactose and the second glucose. After a two day interval collect feces again over a four day period. All feces need not be collected. That contaminated with urine is discarded.

About half a gram (not weighed) of feces from each collection is ashed preferably in a 35 c.c. silica crucible at a moderate temperature. Add a few drops of nitric acid to the residue and heat again to destroy last carbon. Add 10 c.c. of 15 per cent hydrochloric acid and warm until the ash is dissolved. Wash into a flask with water to make about 35 c.c. Ash 2 gm. of food and dissolve ash in the

[1] Bergeim, *Jour. Biol. Chem.*, September, 1926.

same way. Determine iron, calcium and phosphorus[1] in the ash solutions and record the number of mg. of each in 1 c.c. of ash solution.

Calculate the ratios Ca/Fe and P/Fe for food and feces, and calculate percentage absorption or utilization of Ca and P. For example if the ratio Ca/Fe for food is 10:1 and in feces 4:1 unabsorbed Ca is 4/10 or 40 per cent and utilization is 100 − 40 = 60 per cent.

Lactose promotes calcium absorption by creating an acid medium (lactic acid) in the intestines. Glucose has little effect.

4. Influence of Cod Liver Oil on Absorption of Calcium and Phosphorus from Different Portions of the Gastrointestinal Tract. Principle.—The principle is the same as that of the preceding experiment. Animals are killed at the height of

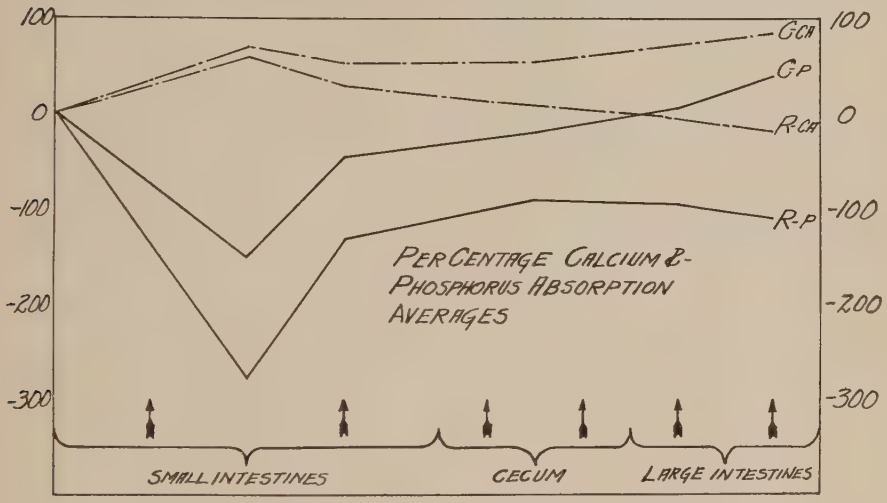


Fig. 84.—Showing Absorption of Calcium and Phosphorus from Different Parts of the Intestinal Tract of Rachitic Rats.

R_1 = untreated rats. C = rats given cod liver oil. Note relative failure of absorption in lower bowel by rachitic animals.

digestion and the percentage of the elements originally present remaining at different levels of the tract determined.

[1] *Calcium Determination.*—To 2 c.c. of ash solution in a 100 c.c. flask add 25 c.c. of water, a drop of alizarin solution and ammonia to neutrality. Add 3 c.c. each of 0.5 N HCl and 2.5 per cent oxalic acid solution. Then add 10 c.c. of 3 per cent ammonium oxalate solution and slowly with shaking 3 c.c. of 20 per cent sodium acetate. Transfer to 50 or 100 c.c. centrifuge tubes (round bottom) let stand for several hours and centrifuge. Draw off supernatant fluid, nearly fill tube with water, invert against palm of hand. Centrifuge, and again draw off fluid. Add 5 c.c. 5 per cent sulphuric acid, warm to 65° and titrate with N/50 potassium permanganate. 1 c.c. equals 0.4 mg. Ca.

Phosphorus Determination.—To 1 c.c. of solution in a large test tube add 10 c.c. of water and 2 drops of concentrated sulphuric acid. Prepare standards in test tubes (2, 4, 7, 10 c.c. of solution containing 0.1 mg. per c.c. of P) adding sulphuric acid and water to make volumes of 11 c.c. Add 2 c.c. of molybdate solution and 2 c.c. of hydroquinone (or 1 c.c. aminonaphtholsulphonic acid) to each tube. Compare in colorimeter. See phosphate methods, Chapter XXXI.

Iron Determination.—To 1 c.c. portions of solution in large test tubes add 8 c.c. of water. Prepare standards (1, 2, 3, 5, and 8 c.c. of ferric sulphate solution containing 0.1 mg. Fe per c.c.) adding to each, 1 c.c. of 1:4 HCl and water to make 9 c.c. Add 2 c.c. 2 per cent KCNS solution to each. Mix and compare. Calculate mg. Fe per c.c.

Procedure.—Feed two or more albino rats (weight 150-200 gm.) on the phosphorus low diet mentioned above for about 25 days. Put in separate cages. Collect feces for a period of 3–4 days. Give one rat 10 drops of cod liver oil and the other 10 drops of olive oil. Continue feeding animals the same diet for 5 days, during the last twelve hours of which the animals are fed every three or four hours. About one gram of food is given at each meal or a little less than required to satisfy the animals' appetites so that the next food will be eaten. Kill animals with chloroform about 4 hours after the last food was given, open the abdomens and remove entire gastrointestinal tract. Separate into stomach, middle small intestine (second and third quarters), lower small intestine (last quarter), cecum and large intestine. Transfer contents of each section to 35 c.c. silica crucibles and ignite (a small amount of $MgCO_3$ may be added to insure against loss of P). Dissolve ash and determine Ca, P, and Fe as in the preceding method. It may be necessary in some cases to dilute the ash solution less and take more solution for determinations.

Calculation.—Calculate percentage absorptions for the different parts of the intestinal tract and plot the results as in Fig. 84. Cod liver oil should increase the absorption of Ca and P the influence being most noticeable in the large intestine. The excretion of phosphoric acid into the upper intestine may be a factor in calcium absorption. A similar procedure may be used for following digestive and other changes in the tract.

CHAPTER XV

PUTREFACTION AND DETOXICATION

The digestive juices as well as the unabsorbed food are subject to the decomposing action of myriads of bacteria. This is particularly true in the large intestine. The result of this action is the production of a large number of more or less toxic compounds from the protein material retained in the intestine.

The symptoms produced by these toxic substances after absorption include general irritability, sleeplessness, loss of appetite, accompanied by a period of melancholia and general mental depression. Intestinal bacteria are found in ever increasing numbers from the pylorus to the anus. The number in the small intestine is however almost insignificant compared with that in the large intestine. The number of bacteria excreted per day has been estimated at from 33 to 128 trillions, and the weight of the dry bacterial cells in certain cases has run as high as 8 grams per day. Probably it is fairly accurate to say that 20–30 per cent of the dry feces is made up of dead bacteria. It is also true that about 50 per cent of the fecal nitrogen is of bacterial origin.

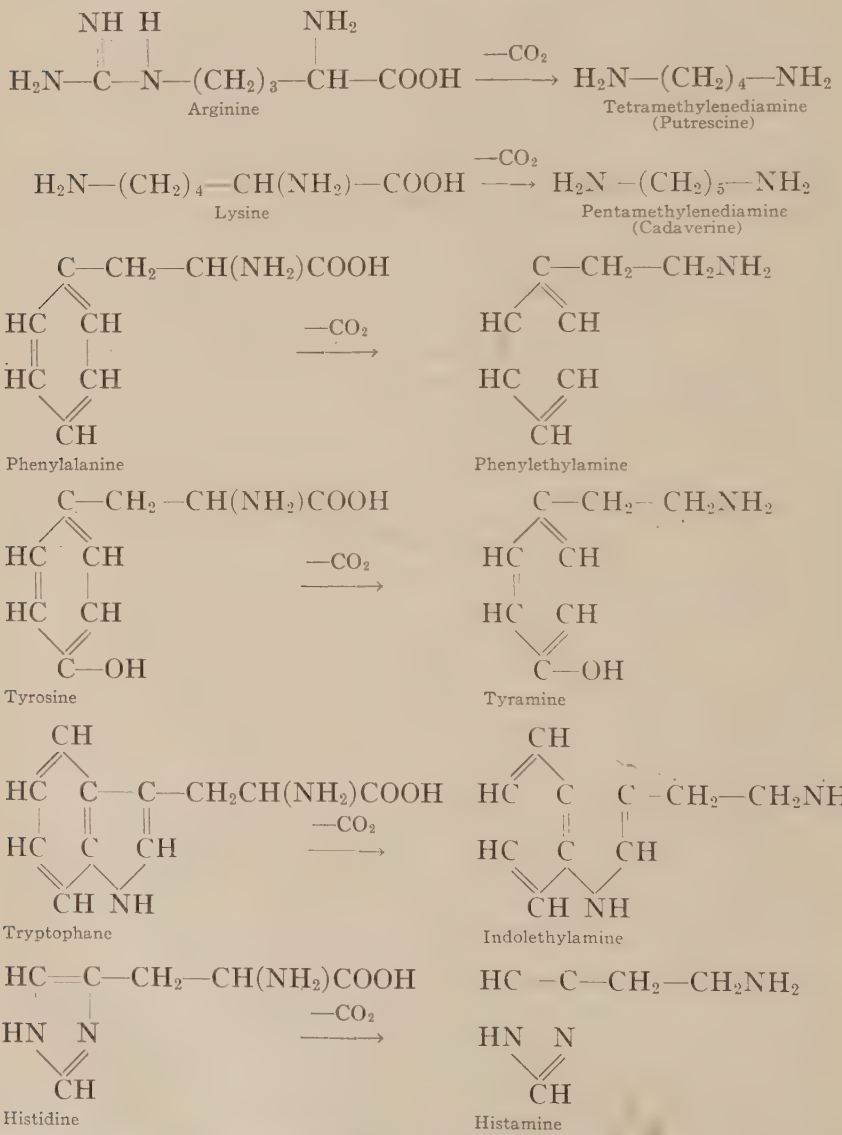
Previously we defined putrefaction as the bacterial decomposition of protein while fermentation was said to be the abnormal disintegration of carbohydrate material in the intestine, but these definitions are no longer tenable, since Ehrlich has shown that amino acids may undergo a process of bacterial decomposition, producing fusel oil or a mixture of alcohols of higher molecular weight than the ordinary ethyl alcohol; that is, bacterial decomposition of proteins ("putrefaction") may yield the same end-products as the bacterial decomposition of carbohydrates ("fermentation").

It is most probable that the bacteria first decompose the protein into α-amino acids by a process much the same as that employed by the proteolytic enzymes in normal digestion; but the amino acids are further decomposed not according to the normal method of deamination and oxidation, but instead lose CO_2 from the carboxyl group (decarboxylation), with the formation of a corresponding alkyl amine or else with a loss of the amino group and conversion into a corresponding fatty acid,

which by subsequent bacterial action may be shortened first by one and later perhaps by several carbon atoms.

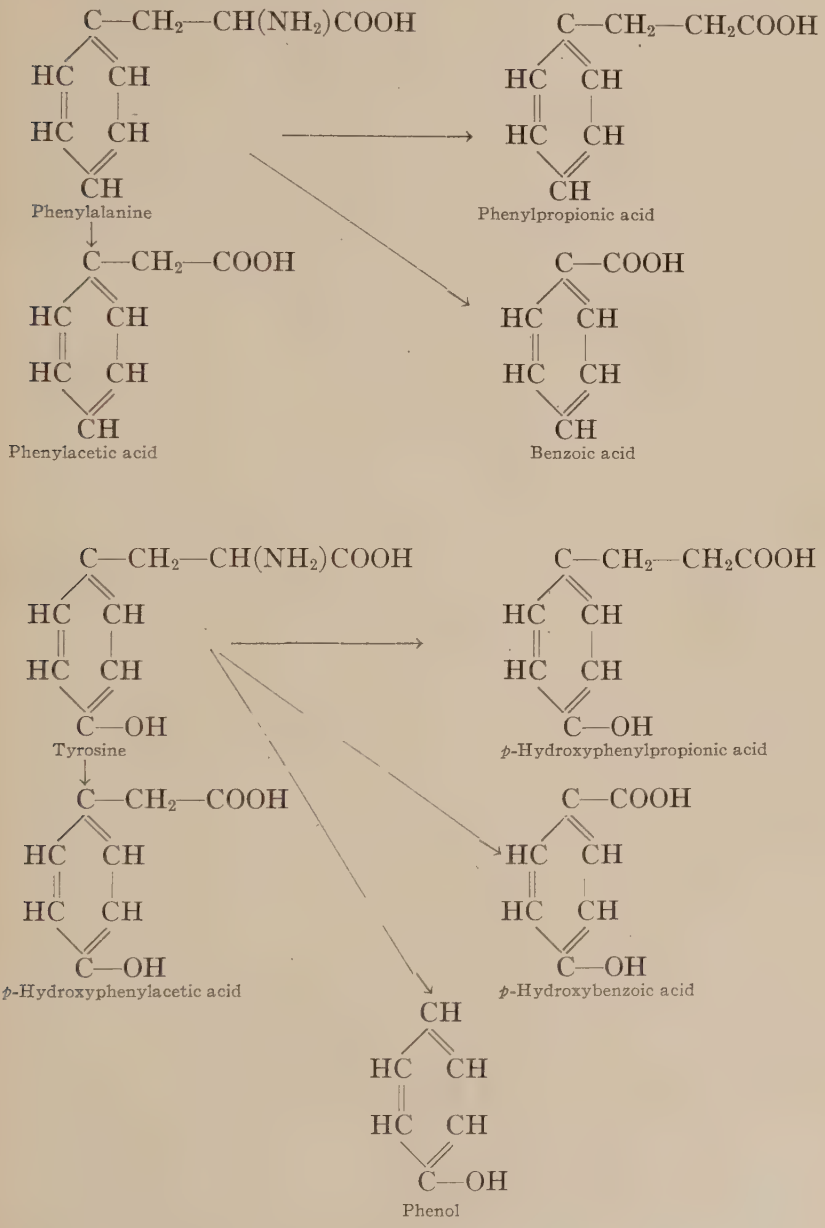
$$R-CH_2-COOH \xleftarrow{-NH_3} R-CH(NH_2)-COOH \xrightarrow{-CO_2} R-CH_2-NH_2$$

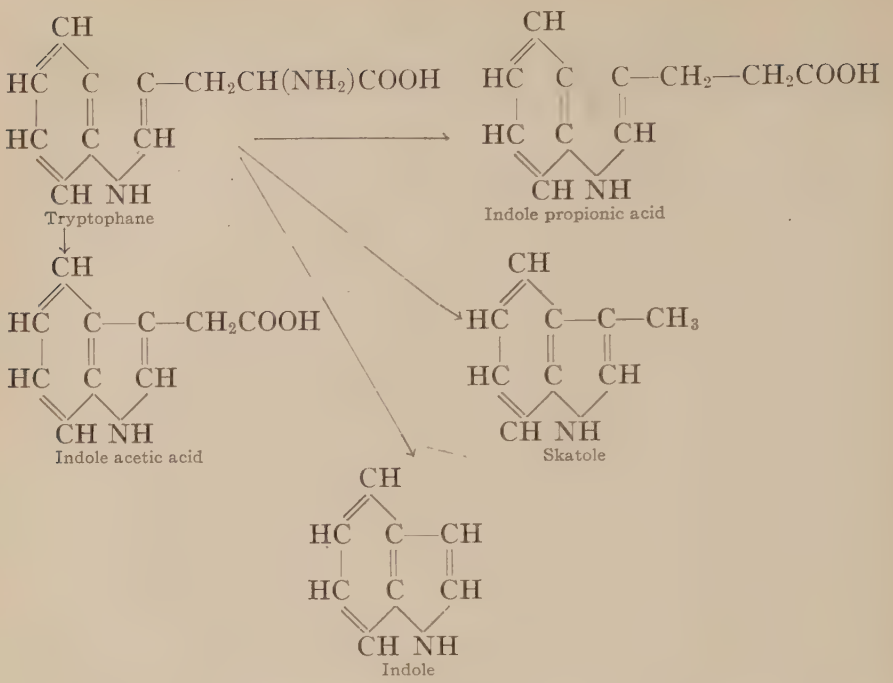
By the action of the carboxylase bacteria on arginine, lysine, phenylalanine, tyrosine, histidine and tryptophane we have the following amines:

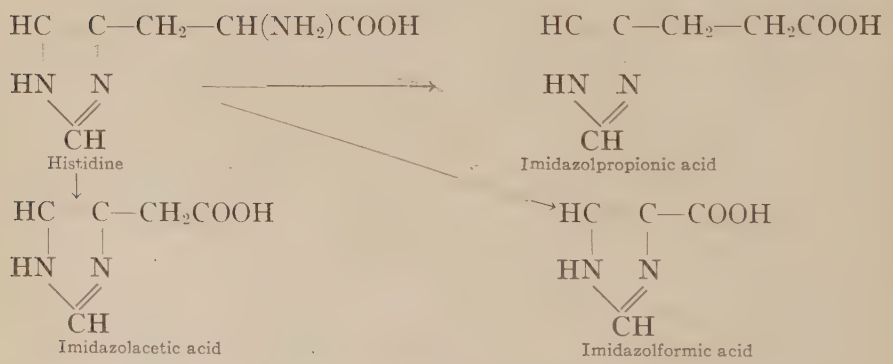


The second type of putrefaction process, namely, the formation of acids, is much more common, but fortunately much less toxic. The

aliphatic amino acids are of little importance in this connection, so that only the more important aromatic amino acids will be represented, showing the progressive steps in their disintegration:







The production of the amine type of putrefactive product by the decarboxylation of the amino acids may be brought about by a great variety of organisms, especially anaerobic bacilli. This power however seems to be most pronounced in the *bacillus aminophilus intestinalis*. Koessler and Hanke[1] have shown that the formation of histamine is always coincident with a medium which is distinctly acid. They believe that histamine is produced by the *bacillus coli communis*, to neutralize the excess of acidity. However, not all strains of this bacillus are able to produce histamine from histidine, as these authors found only

[1] Koessler and Hanke, *Jour. Biol. Chem.*, 39, 538, 1919.

six strains able to bring about this reaction out of the twenty-nine which they tried.

This nitrogenous type of putrefaction product[1] is much more potent in its physiological action than the non-nitrogenous variety. The diamino derivatives of the amino acids have however been considerably over-estimated as to their clinical importance. Pentamethylene diamine (cadaverine) and tetramethylene diamine (putrescine) cause a *decrease* rather than an increase in blood pressure. The mono-amino derivatives however when injected directly into the blood stream have a powerful action on unstriped muscle, giving an immediate and very pronounced effect on arterial tension; even a few milligrams of the amine cause an alarming increase. Tyramine along with ergotoxine constitutes the active principle of the pharmaceutical preparation ergot, which has a most pronounced effect on the sympathetic nervous system, causing the nonpregnant uterus to contract. All the glands which are innervated by the sympathetic system are also stimulated to secretion. Both tyramine and histamine have at various times been popularly associated with arterial hypertension.

It must be remembered that none of these amines has as yet been proved to be a direct etiological factor in disease. Fortunately they are found in exceedingly small amounts even in marked constipation. When they are formed they are, like all other such products "filtered" through the intestinal wall, picked up by the portal circulation and passed through the liver before they are thrown into the general circulation. It has been shown definitely by Guggenheim and Loeffler[2] also by Sherwin and co-workers[3,4] that these amines may be introduced directly into the gastro-intestinal tract in amounts much greater than could be found in severe constipation, without the development of toxic symptoms. Furthermore the detoxicated products could be isolated from the urine of the subject. Koessler and Hanke have shown that the intestinal wall plays a very important part at least in the detoxication of histamine. Apparently the amine is first hydrolyzed to the corresponding alcohol, next oxidized to the aldehyde and then to the acid. This acid may then be joined with a second molecule if necessary to decrease further its toxicity.

$$R-CH_2-CH_2NH_2 + H_2O \xrightarrow{-NH_3} R-CH_2-CH_2OH \xrightarrow{+O} R-CH_2-CHO \xrightarrow{+O} R-CH_2-COOH$$

[1] Guggenheim, Die Biogene Amine, Julius Springer, Berlin, 1920.
[2] Guggenheim and Loeffler, *Biochem. Zeit.*, 72, 352, 1916.
[3] Novello, Wolf and Sherwin, *Amer. Jour. Med. Sci.*, 170, 888, 1925.
[4] Power and Sherwin, *Arch. Int. Med.* (In press.)

Of the second type of putrefaction products, in which the amino group is first eliminated, the toxicity is rather low, and detoxication is usually complete. Only a few of these products (see pp. 292–294), are worthy of special mention: benzoic acid, because it is practically always present in the urine in the form of its detoxication product, hippuric acid; phenol, as it always occurs, either combined or uncombined in the urine; and lastly, indoxyl and skatoxyl, the two abnormal disintegration products of tryptophane, which are of considerable historical importance, and probably the chief factors in producing the "bowel breath," nausea and headache, so commonly experienced in cases of constipation. In fact, all these symptoms can be artificially produced, if one ingest as much as 0.05 gram of either indole or skatole,[1] which should be enclosed in a gelatin capsule and thoroughly covered with salicin, to insure its passage through the stomach. The capsule empties its contents by this method in the small intestine, instead of in the stomach, but even with these precautions, the material is regurgitated into the stomach, and the odor appears in the breath for six to eight hours.

The older quantitative tests for putrefaction in the intestine have proved to be only approximate. The indican tests are not quantitative, as they fail to give even comparative values, and at best would be indicators only for those proteins containing tryptophane. The ethereal sulphate output cannot be taken as an index of putrefaction, as this form of detoxication is limited mainly to the endogenous sulphur catabolism, and hence is too easily exhausted. The recent reduction test of Bergeim,[2] described on page 306 appears to be most promising.

Detoxication.—Under the term detoxication[3] we include all those chemical changes taking place in the animal organism which have a tendency to render less harmful any poisonous compound or to aid in its rapid elimination. Berczeller[4] believes that the toxicity of a given substance is proportional to the lowering of surface tension which it produces in the body fluids. Rose and Sherwin[5] find in many cases that the detoxication product lowers the surface tension of a solution less than the toxic substance itself, but that this is by no means always the case. Three types of detoxicating action are most common: oxidation, reduction and combination or conjugation. It must be remembered also that different species of animal may employ entirely different types of reaction. For example, phenylacetic acid is combined with

[1] Novello, Wolf and Sherwin: *Loc. cit.*
[2] Bergeim: *Jour. Biol. Chem.*, 62, 45, 1924.
[3] For review see Sherwin: *Physiol. Rev.*, 2, 238, 1922.
[4] Berczeller: *Bioch. Zeit.*, 84, 75, 1917.
[5] Rose and Sherwin: *Jour. Biol. Chem.*, 68, 565, 1926.

glutamine[1] by the human being, with glycocoll by dog, cat, rabbit, monkey[2] and other lower animals, but with ornithine by the fowl.[3] p-Aminobenzoic acid[4] is detoxicated in the dog by a combination with glycocoll through the carboxyl group of the acid, while the human being and rabbit detoxicate this same compound by conjugating acetic acid with the amino group of the aminobenzoic acid.

Oxidation.[5]—The first attempt of the body is the complete destruction of a toxic molecule through oxidation. This is more or less successful in the case of aliphatic compounds, but is of little value in the destruction of aromatic compounds. In the latter cases it is possible to oxidize one and only one side chain to a side chain consisting of only one or two carbon atoms; thus, phenylvaleric acid is by means of β-oxidation shortened to benzoic acid, while phenylbutyric acid is oxidized to phenylacetic acid.

$$\underset{\text{Phenylvaleric acid}}{C_6H_5CH_2CH_2CH_2CH_2COOH} \rightarrow \underset{\text{Phenylpropionic acid}}{C_6H_5CH_2CH_2COOH} \rightarrow \underset{\text{Benzoic acid}}{C_6H_5COOH}$$

$$\underset{\text{Phenylbutyric acid}}{C_6H_5CH_2CH_2CH_2COOH} \rightarrow \underset{\text{Phenylacetic acid}}{C_6H_5CH_2COOH} + \underset{\text{Acetic acid}}{CH_3COOH}$$

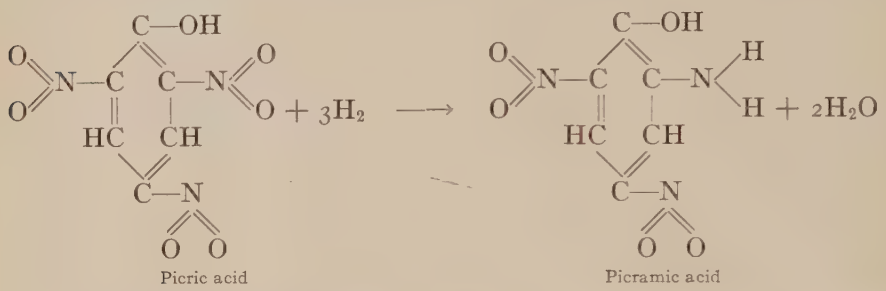
The process of β-oxidation is thus employed as in the normal metabolism of fats. (See Chapter XXXII.)

Reduction.[5]—Reduction is much less common than oxidation, but nevertheless the intestines of man and animals are the seat of powerful reduction processes. The biliverdin and bilirubin of the bile are here reduced to urobilin and still further to the colorless urobilinogen. Bismuth salts are reduced to the suboxide and ferric salts to the ferrous condition. Pigments such as methylene blue and litmus are decolorized. Sulphur and even sulphates may be reduced to hydrogen sulphide. This strongly reducing character of the intestinal contents cannot be without influence on some of the many chemical reactions occurring within the intestines. The oxygen supply must be of particular importance for the bacteriology of the intestine, the removal of oxygen by reducing substances presumably favoring the growth of obligate anaerobes, among which are organisms of putrefaction concerned in the formation of those products whch are considered chiefly responsible for the toxemias of intestinal origin. The reduction processes occur almost entirely in the lower bowel and are increased by diets which increase intestinal putrefaction. The extent of the reduction processes

[1] Thierfelder and Sherwin: *Ber.*, 48, 2630, 1914.
[2] Sherwin: *Jour. Biol. Chem.*, 36, 309, 1918.
[3] Jaffe, *Ber.*, 10, 1925, 1877.
[4] Muenzen, Cerecedo and Sherwin: *Jour. Biol. Chem.*, 67, 469, 1926.
[5] Oxidations and Reductions in the Animal Body, H. D. Dakin, Monograph, Longmans. London, 1922.

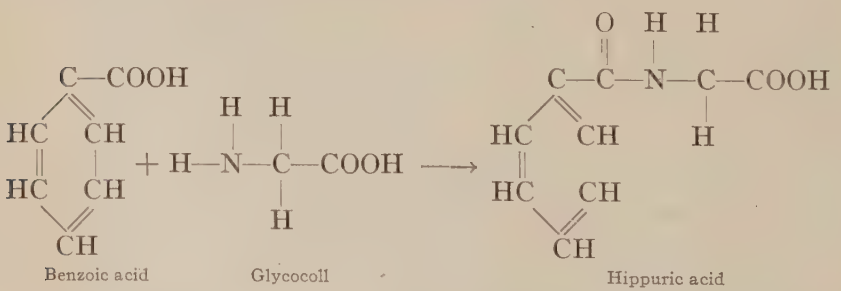
may be measured and is an index of intestinal putrefaction. The power of forming reducing substances is not limited to any one type of organism. It is dependent upon the rapidity of growth of the organisms. Acid fermentations in the small intestines result in but slight reduction of iron. Bergeim's procedure (see p. 306) provides a means of estimating intestinal reductions.

The transformation of picric acid to picramic acid is an important example of a reduction process.[1]



Combination or Conjugation.— When oxidation or reduction fails to destroy completely a toxic substance, the part which cannot be oxidized is joined to another compound or radical, which appears to make it less toxic and at the same time more soluble, thus hastening its elimination in the body excretions. The following substances are known to be available: glycocoll, glutamine, cysteine, ornithine, glycuronic acid, sulphuric acid, acetic acid and the methyl group.

Glycocoll or glycine is used for the detoxication of more than one hundred known organic acids. The best known reaction is its combination with benzoic acid to form hippuric acid.

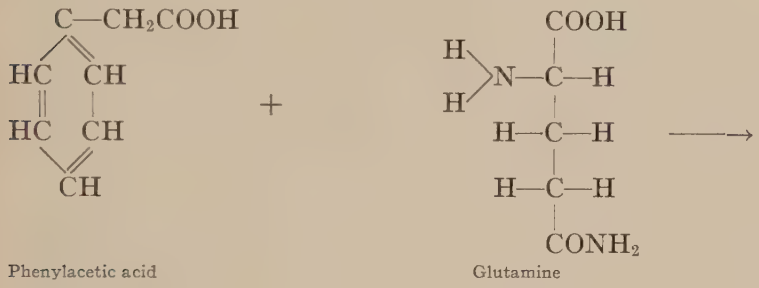


Glycocoll can be synthesized in the human[2] as well as in the animal body[3] for the detoxication of benzoic acid. It is produced at the expense of the nitrogen which otherwise would have been excreted as

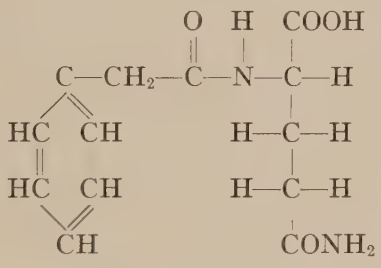
[1] Rose, Novello and Sherwin, unpublished data.
[2] Shiple and Sherwin: *Jour. Amer. Chem. Soc.*, 44, 618, 1922.
[3] McCollum and Hoagland: *Jour. Biol. Chem.*, 16, 311, 1913.

urea. This is accomplished in man, after a 5 gram dose of benzoic acid, without increasing the destruction of body tissue, although the subject is maintained on a non-protein diet.

Glutamine is used by the human being for the detoxication of phenylacetic acid.[1] It has not been observed in the detoxicating mechanism of any of the lower animals. It is synthesized by the human being in the same manner as glycocoll.[1] The human being is able to synthesize both glycocoll and glutamine simultaneously after ingesting a 5 gram dose of phenylacetic acid and benzoic acid.

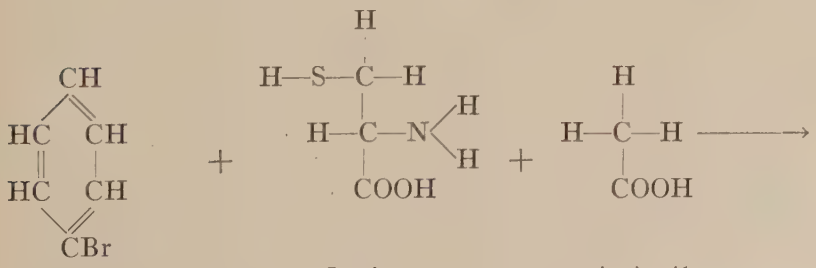


Phenylacetic acid Glutamine



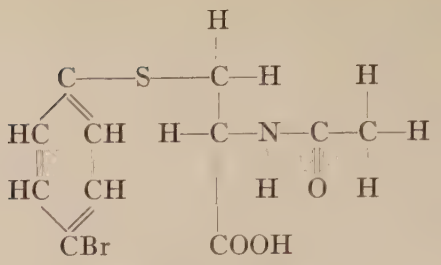
Phenylacetylglutamine

Cysteine is employed by a number of animals, particularly the dog,[2] for the detoxication of brombenzene, with a subsequent acetylation of amino group of the cysteine.



Brombenzene Cysteine Acetic acid

[1] Sherwin, Wolf and Wolf: *Jour. Biol. Chem.*, 37, 113, 1919.
[2] Jaffe: *Ber.*, 12, 1092, 1879.

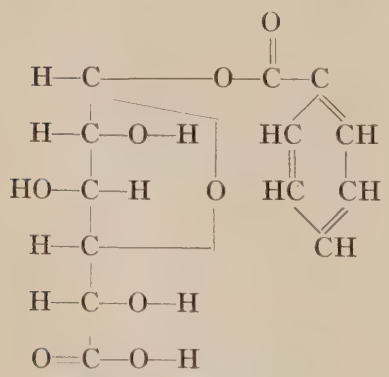


Bromphenylmercapturic acid

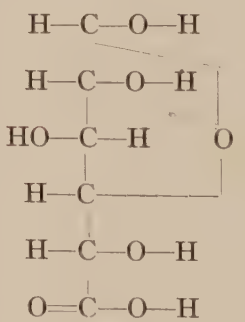
This so-called mercapturic acid reaction apparently does not take place in the human body, nor is it employed by the rabbit. Cysteine *cannot be synthesized* for detoxication purposes in the animal organism, even though various forms of sulphur and nitrogen are available for the synthesis,[1] but only when cystine or cysteine is included in the diet is it possible for the animal to form mercapturic acid.

Ornithine is employed by the fowl for the detoxication of benzoic acid.[2] This can be synthesized in the organism of the fowl from the uric acid fraction instead of the urea fraction,[2] as the waste nitrogen in the case of the fowl is excreted as uric acid and not as urea.

Glycuronic acid is employed by all species of animals for the detoxication of aliphatic or aromatic alcohols, when a glucoside or ether type of binding occurs. It may also be used for the detoxication of aromatic acids like benzoic acid, when an ester-like linkage results.

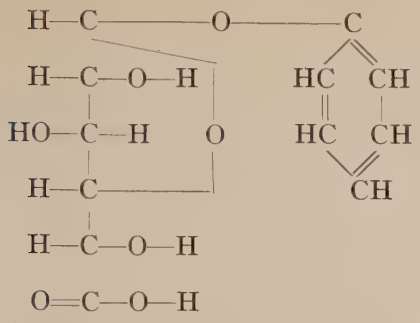


Benzoylglycuronate (ester type of binding)



Glycuronic acid

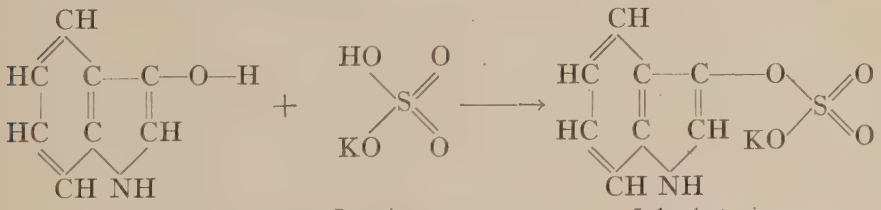
[1] Muldoon, Shiple and Sherwin: *Jour. Biol. Chem.*, 59, 675, 1924.
[2] Crowdle and Sherwin: *Jour. Biol. Chem.*, 55, 365, 1923.



Phenolglycuronate (glucide or ethereal type)

Glycuronic acid is apparently synthesized in the animal organism, but the source of the substance is still a mystery. It may be one of the intermediary products of glucose metabolism, but there is no available data to prove this assumption. A rabbit fed menthol will excrete large amounts of menthol glycuronic acid,[1] but this amount is no more affected by the simultaneous feeding of glucose than it is by the feeding of amino acids, since both increase the output of the glycuronate. Peculiarly, the amino acids which form no glucose in the body have the same effect as the glucose formers.[2] Friedmann and Koechig[3] believe that there is a definite relation between the amount of carbohydrate stored in the tissues and glycuronic acid synthesis.

Sulphuric acid like glycuronic acid is employed principally in the detoxication of hydroxyl or alcoholic compounds. These conjugated sulphates compose that sulphate fraction of the urine known as the "ethereal sulphates." The second replaceable hydrogen of the sulphuric acid is usually neutralized with sodium or potassium. Thus indoxyl and skatoxyl (the oxidation products of indol and skatol respectively) are excreted as ethereal sulphates or glycuronates.



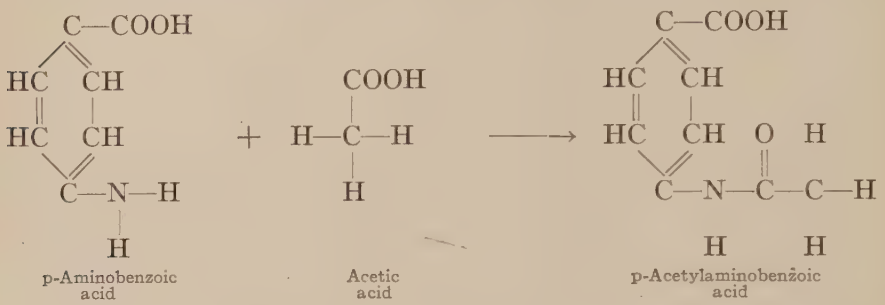
Indoxyl | Potassium acid sulphate | Indoxylpotassium sulphate or Indican

The etheral sulphates are formed largely at the expense of endogenous catabolism,[4] hence are always limited in amount. For this reason also

[1] Quick: *Jour. Biol. Chem.*, 61, 679, 1924.
[2] Sherwin and co-workers, unpublished data.
[3] Friedmann and Koechig: *Proc. Soc. Exp. Biol. and Med.*, 33, 369, 1926.
[4] Shiple, Muldoon and Sherwin: *Jour. Biol. Chem.*, 60, 59, 1924.

the quantity of ethereal sulphates in the urine *should not* be considered as an index of intestinal putrefaction.

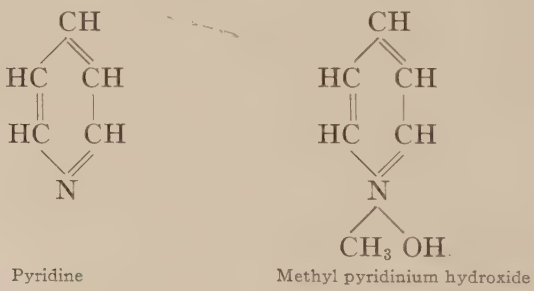
Acetic acid up to the present time has been found only in the detoxication of amino compounds. Examples of this are the detoxication of p-aminophenylacetic acid[1] and p-aminobenzoic acid by acetic acid in the human organism and also in the rabbit.[2]



p-Aminobenzoic acid — Acetic acid — p-Acetylaminobenzoic acid

Acetic acid is not stored in the tissues, but is synthesized in the body, probably from the products of intermediary fat metabolism and those amino acids which produce acetone bodies instead of glucose in the diabetic organism.

The methyl group is joined to a very limited number of compounds, of which pyridine is a good example.



Pyridine — Methyl pyridinium hydroxide

Nothing is known regarding the source of the methyl group. Methylation is always attended by considerable destruction of body tissue, probably due to the marked toxicity of the few pyridine derivatives which are methylated.[3]

It must be remembered that while the defense mechanism against putrefaction products is by no means specific nor unlimited in its application, as Wells[4] has pointed out, yet it is more than capable of detoxi-

[1] Cerecedo and Sherwin: *Jour. Biol. Chem.*, 58, 215, 1923.
[2] Muenzen, Cerecedo and Sherwin: *Jour. Biol. Chem.*, 68, 469, 1926.
[3] Sherwin and co-workers, unpublished data.
[4] Wells: Chemical Pathology, 5th edition, Saunders and Co., p. 262, 1925.

cating the quantity of toxic substances found under even very abnormal conditions. Where these reactions take place is still a matter of doubt. The formation of hippuric acid apparently takes place chiefly in the kidney, but to some extent in the liver; histamine[1] is detoxicated chiefly in the intestinal wall. The acetylation reactions have been shown by Vesell and Sherwin[2] to take place in the liver, and to be an index of hepatic function.

Experiments on Putrefaction, Detoxication and Reduction

TESTS FOR PRODUCTS OF PUTREFACTION

Indole and Skatole

1. Herter's β-Naphthaquinone Reaction.—To 10 c.c. of unknown solution add 2 drops of 2 per cent solution of β-naphthaquinone sodium monosulphonate and 2 c.c. of 10 per cent NaOH. Let stand 15 minutes. Shake with 2 c.c. of chloroform. A pinkish red color in the chloroform indicates indole. This is a very delicate test. For quantitative application see p. 328.

2. Ehrlich's p-Dimethylaminobenzaldehyde Reaction.—To 10 c.c. of unknown solution add 1 c.c. of 5 per cent alcoholic solution of p-dimethylaminobenzaldehyde and 1 c.c. of concentrated HCl. Indole gives a red color and skatol a blue color.

3. Nitroso-indole Reaction.—Acidify the mixture with nitric acid and add a few drops of KNO_2 solution. Indole gives a red color and skatole a white turbidity.

4. Herter's p-Dimethylaminobenzaldehyde Reaction.[3]—To 5 c.c. of solution add 1 c.c. of 5 per cent solution of p-dimethylaminobenzaldehyde in 10 per cent sulphuric acid and heat to boiling. A purplish blue color indicates skatole.

Phenols, Hydroxy Aromatic Acids and Imidazoles

1. Hanke and Koessler's p-Diazobenzenesulphonic Acid Reactions.[4]—These tests are used for the detection and quantitative determination of histidine, histamine and other imidazoles (giving a pink color), phenol, *o*- and *m*-cresol (yellow color), *p*-cresol, *p*-oxyphenylpropionic, *p*-oxyphenylacetic and *p*-oxyphenyllactic acids (red color) and tyrosine and tyramine (bluish red on adding hydroxylamine).

To 5 c.c. of 1.1 per cent sodium carbonate solution add 2 c.c. of p-diazobenzenesulphonic acid reagent.[5] After 1 minute add 1 c.c. of solution to be tested

[1] Koessler and Hanke: *Jour. Biol. Chem.*, 59, 889, 1924.
[2] Vesell and Sherwin: *Arch. Int. Med.*, 37, 257, 1926.
[3] Herter: Bacterial Infections of the Digestive Tract, 1907.
[4] Hanke and Koessler: *Jour. Biol. Chem.*, 39, 497, 1919; 50, 235 and 271, 1922; 59, 803–921, 1924; 66, 475, 1925.
[5] *p-Diazobenzenesulphonic Acid Reagent.* Measure 1.5 c.c. each of the stock sulphanilic acid (4.5 gm. mixed with 45 c.c. 37 per cent HCl and made to 500 c.c.) and sodium nitrite (25 gm. of 90 per cent product made to 500 c.c.) solutions into a 50 c.c. volumetric flask. Immerse in ice bath for 5 minutes. Then add 6 c.c. more of nitrite solution. Mix and replace in ice bath for 5 minutes. Dilute to mark. Keep on ice. Use after 15 minutes. Prepare fresh each day.

and mix. Note color and rapidity with which it develops. For tyrosine and tyramine make strongly alkaline with NaOH and then add a small amount of hydroxylamine hydrochloride solution. For details of separation and determination, see original papers.

2. Millon's Test.—Test the material with Millon's reagent. A red color is given by compounds containing the hydroxyphenyl group.

3. Bromine Test.—Add a few drops of bromine water. Cresols, phenol, and hydroxy aromatic acids give white precipitates of bromine derivatives.

PREPARATION OF PHENYLACETYLGLUTAMINE

$C_6H_5.CH_2.CONH.CH(COOH)CH_2.CH_2.CONH_2$

Although theoretically this compound could be made by a coupling reaction between phenylacetylchloride and glutamine, yet owing to the difficulty of obtaining the latter the compound is best obtained as the detoxication product of phenylacetic acid by the human organism.

Ingest 10 grams of phenylacetic acid in two 5 gram doses taken 24 hours apart, preferably after a meal. The substance is ingested as the water solution of its sodium salt, made faintly acid with hydrochloric acid. Collect the urine until 24 hours after the second dose and without adding any substance to it evaporate it on the water bath. Do not take to absolute dryness, but stop when the residue in the dish begins to froth. Make slightly acid to Congo red with 25 per cent sulphuric acid, and after thorough cooling add cautiously about 10 c.c. of concentrated sulphuric acid, mixing vigorously to avoid local overheating. Transfer the pasty mixture to a rotary extractor (Fig. 85) and extract from one to three hours with absolute ethyl acetate.[1] When the boiling ethyl acetate in the flask of the extraction apparatus begins to precipitate a brownish gum, disconnect the flask and quickly filter the liquid into a beaker immersed in ice water. Stir vigorously and let stand for some time in the cold. The mass of white crystals is then filtered off on a Buchner funnel and washed with cold ethyl acetate. The extraction may be repeated a number of times, each time taking out a little more of the desired substance, until so little crystallizes out that it does not pay to continue. The white crystalline precipitate is the molecular addition compound of urea with phenylacetylglutamine, mixed sometimes with free urea. The free urea and that combined with the substance are both removed together by means of urease; this serves to determine the amount of urea present, but is not satisfactory for the isolation of the pure substance.

Pure phenylacetylglutamine may be obtained from the urea addition compound as follows:[2] Dissolve the substance in water and make slightly alkaline with barium hydroxide. Do not warm the solutions. Remove the excess barium with carbon dioxide, filter, and evaporate at not too high a temperature on the water bath. The resulting mixture of urea and the barium salt of phenylacetylglutamine is dried thoroughly in a vacuum oven at about 60° and is then extracted three times with absolute alcohol, cooling after each extract, and filtering off the alcoholic solution of urea. The residue, i.e., the pure barium salt of

[1] The anhydrous ethyl acetate of the Eastman Kodak Co. or of the U. S. Industrial Chemical Co. is satisfactory for this purpose. Ordinary commercial ethyl acetate containing appreciable quantities of water and alcohol is useless.

[2] Sherwin, Wolf, and Wolf: *Jour. Biol. Chem.*, 37, 115, 1919.

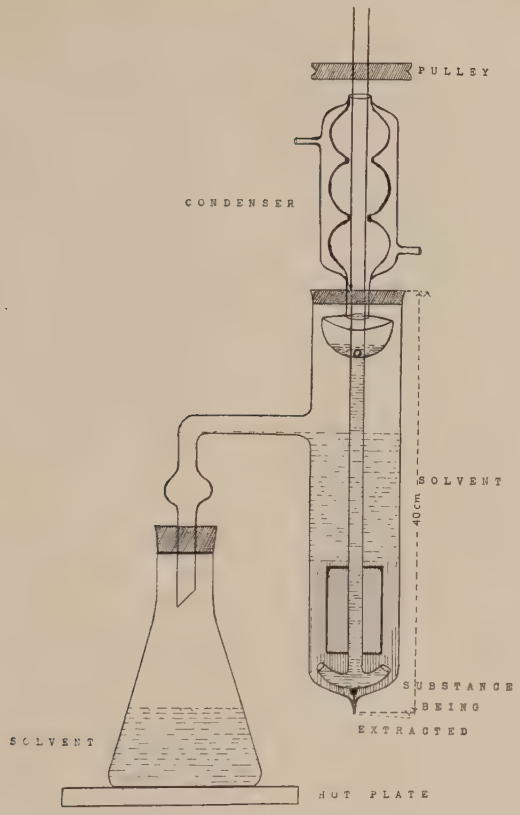


FIG. 85.—ROTARY EXTRACTOR.[1]

[1] The condenser may be attached to the body of the extractor by means of a cork stopper or by a ground joint. The long glass tube running down through the condenser and extractor opens out into a sort of curved T at the bottom and rotates on a platinum pin as bearing, fitting into the bottom of the extractor. Above this T, two glass rods bent into rectangular shape are sealed on to act as stirrers. Inside the body of the extractor and directly under the condenser a sort of glass cup is sealed on to the central glass stem, which has a small hole blown in it above its junction with the cup. The solvent is boiled on the hot-plate, and the vapor on condensing runs into the cup and thence down the stem through the small hole. After a sufficient column of solvent has backed up in the stem to overbalance the pressure of the material being extracted (which should be of a pasty consistency) pure solvent will begin to flow out of the openings at the bottom of the stem and will carry some dissolved substances up with it. The excess volume thus introduced into the extractor proper will then overflow back into the flask; so the liquid in the flask will continually be getting richer in the substances being extracted, while the general form of the apparatus prevents much of the "muck" in the bottom of the extractor from getting into the flask. The stem carries a pulley at the top (a flat cork stopper with a groove in it will do) and by means of a suitable speed reduction device is driven by a motor. The stem should make about 60–80 revolutions per minute.

phenylacetylglutamine, is then dissolved in the smallest possible quantity of water, the calculated amount of phosphoric acid is added, and the barium phosphate filtered off. The aqueous solution is then concentrated at room temperature in vacuo and extracted with absolute ethyl acetate, yielding pure phenylacetylglutamine.

Estimation of Intestinal Reductions. Method of Bergeim.[1] Principle.—A definite amount of ferric oxide is mixed with the food. In the feces the percentage of the oxide which has been reduced is determined. This is an index of the intensity of reduction processes in the bowel.

Procedure.—Experiments may be made on albino rats. The animals under investigation are given any desired diet with which the iron salt used is incorporated to make a uniform mixture. A mixture with 1 per cent of hydrated ferric oxide powder (completely soluble in 100 parts of 12 per cent hydrochloric acid in about 30 seconds on the boiling water bath) or one-fourth this amount of a coarser ferric oxide (dissolving in about 2 minutes) may be used. The same preparation should be used throughout a given series of experiments.

The following diets may be tested:

(1) Starch 80, agar 1, ferric oxide 1, casein 20.
(2) Egg albumin instead of casein.
(3) No. 2 and 50 per cent of glucose.
(4) No. 2 and 50 per cent of lactose.
(5) Milk powder 98, agar 1, ferric oxide 1.
(6) Meat powder 98, agar 1, ferric oxide 1.

After 2 days on the experimental diet specimens of feces are collected. In the case of the rat these may be obtained by digital pressure on the lower bowel.

About 0.2 gm. of feces (not weighed) is rubbed up with 10 c.c. of dilute HCl (1:2) and the mixture heated in a test tube of about 18 mm. diameter on a boiling water bath for ½ minute (2 minutes if the coarser iron oxide is used). If biliary or other pigment be present in appreciable amount 0.5 gm. of acid-extracted blood charcoal should be added before heating. This is usually not necessary in studies on rats, but is required in the examination of human feces. The tubes are placed in cool water for about ½ minute and then filtered preferably within ½ hour. 2 or 3 c.c. aliquots of the filtrate are measured into each of two 25 c.c. graduated cylinders. To one add enough 0.1 N potassium permanganate solution to give a pink color lasting for about 1 minute (1 or 2 c.c. are usually required). This oxidizes the ferrous iron to the ferric condition. Then to each cylinder add 2 or 3 c.c. of a 1 per cent solution of potassium thiocyanate and make each to volume (usually 7 to 10 c.c.). Compare the two solutions obtained in a colorimeter.

Calculation.—Divide the lower by the higher reading, multiply by 100, and subtract from 100. This gives the percentage of ferric oxide reduced.

Interpretation.—Reduction is brought about primarily by bacterial action in the cecum and large intestine. Casein diets show much less reduction than those containing equal amounts of egg or meat protein. Lactose given in fairly large amounts, because of its slow digestion and absorption, reaches the lower bowel and is there fermented to produce lactic acid which inhibits the growth of putrefactive organisms and lessens reduction. Milk is therefore particularly effective in decreasing intestinal putrefaction and reductions.

[1] Bergeim: *Jour. Biol. Chem.*, 64, 45, 1924.

Reductions by Intestinal Bacteria. Test Tube Experiments.—Rub up about 50 gm. of intestinal contents or feces with an equal volume of water and 5 c.c. of 5 per cent dialyzed iron to which enough sodium carbonate solution has been added to neutralize the acidity. Strain through cheese cloth. Introduce 10 c.c. of the mixture into each of six test tubes.

To No. 1 and No. 2 add about 0.5 gm. powdered sulphur and to Nos. 3 and 4 about 0.5 gm. of powdered calcium sulphate. Add paraffin oil to each tube. Heat Nos. 2, 4, and 6 in a boiling water bath for 10 minutes to kill bacteria. Incubate all tubes over night at 40°. Note which tubes show the greatest reduction of the ferric hydroxide as indicated by darkening due to ferrous oxide.

Introduce 2 c.c. of each mixture into a series of test tubes equipped for aspiration. Add 1 c.c. of concentrated HCl to each and aspirate into a series of test tubes each containing 5 c.c. of a solution of gelatin 0.5 per cent, NaOH 0.5 per cent and lead acetate 0.05 per cent. A brown or black suspension of lead sulphide is formed whose density is proportional to the amount of sulphide present. Is any of the sulphur or sulphate reduced to hydrogen sulphide by bacteria present? The bacterial processes occurring in feces for the first 24 hours after passage are believed to be similar to those taking place in the bowel. After this the bacterial action may assume a different character.

CHAPTER XVI

FECES

THE feces are the residual mass of material remaining in the intestine after the full and complete exercise of the digestive and absorptive functions and are ultimately expelled from the body through the rectum.

They may be said to be composed of the following substances:

1. Food residues: (*a*) those portions of the food which have escaped absorption, and (*b*) that part of the diet either not digested or incapable of absorption.

2. The remains of the intestinal and digestive secretions not destroyed or reabsorbed.

3. Substances excreted into the intestinal tract, notably salts of calcium, iron, and other metals.

4. The bacterial flora of the intestinal tract.

5. Cellular elements to which may be added, under pathological conditions, blood, pus, mucus, serum, and parasites.

6. Abnormally: enteroliths, gall stones, and pancreatic calculi.

The *amount* of the fecal discharge varies with the individual and the diet. Upon an ordinary mixed diet various authorities claim that the daily excretion by an adult male will aggregate 110–170 grams with a solid content ranging between 25 and 45 grams; the fecal discharge of such an individual upon a vegetable diet will be much greater and may even be as great as 350 grams and possess a solid content of 75 grams. In the authors' experience the average daily output of moist feces, calculated on the basis of data secured from the examination of over 1000 stools, was about 100 grams. The variation in the normal daily output being so great renders this factor of very little value for diagnostic purposes, except where the composition of the diet is accurately known. Lesions of the digestive tract, a defective absorptive function, or increased peristalsis as well as an admixture of mucus, pus, blood, and pathological products of the intestinal wall, may cause the total amount of excrement to be markedly increased. An idea of the variation of the percentage of dry matter in the feces, evacuated after the ingestion of different diets, may be gathered from a consideration of the following table.[1]

[1] Schmidt and Strasburger: "Die Fäzes des Menschen," Berlin, 1915.

INFLUENCE OF DIET ON FECAL DRY MATTER

Diet		Dry Matter Percent.
Milk	Nursing infant	15.0
	Adult	28.0
Meat		29.0
Bread		25.0
Potatoes		15.0
Cabbage		4.4
Mixed Diet		26.0

The fecal *pigment* of the normal adult is hydrobilirubin. This pigment originates from the bilirubin which is secreted into the intestine in the bile, the transformation from bilirubin to hydrobilirubin being brought about through the activity of certain bacteria. Hydrobilirubin is sometimes called stercobilin and bears a close resemblance to urobilin or may even be identical with that pigment. Neither bilirubin nor biliverdin occurs normally in the fecal discharge of adults, although the former may be detected in the excrement of nursing infants. If these pigments are found in the feces of adults, they indicate an abnormally rapid transit through the large bowel thus preventing their transformation into hydrobilirubin. Frequently, in some way as yet unknown, probably through the agency of certain bacterial processes, colorless hydrobilirubinogen (leucohydrobilirubin) is formed which after the passage of the movement and exposure to air is reconverted into hydrobilirubin. This may explain in some cases the darkening of the stool when exposed to the air. The most important factor in determining the color of the fecal discharge is the diet. A mixed diet, for instance, produces stools which vary in color from light to dark brown, an exclusive meat diet gives rise to a brownish-black stool, whereas the stool resulting from a milk diet is invariably light colored. Certain pigmented foods, such as the chlorophyllic vegetables and various varieties of berries, each afford stools having a characteristic color. Certain drugs act in a similar way to color the fecal discharge. This is well illustrated by the occurrence of green stools following the use of calomel, of black stools after bismuth ingestion, and of yellow stools

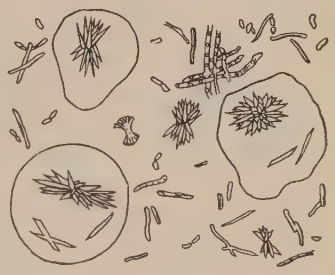


FIG. 86.—HEMATOIDIN CRYSTALS FROM ACHOLIC STOOLS. (*v. Jaksch.*)
Color of crystals same as the color of those in Fig. 80, page 276.

following the administration of rhubarb, senna or santonin. The green color of the calomel stool is generally believed to be due to biliverdin. v. Jaksch, however, claims to have proven this view to be incorrect since he was able to detect hydrobilirubin (or urobilin) but *no biliverdin* in stools after the administration of calomel. The bismuth stool was at one time thought to derive its color from the black sulphide which is formed from the subnitrate of bismuth. We now know[1] that the color is due to the reduction of the bismuth compound (subnitrate) to *bismuth suboxide*. In cases of biliary obstruction the grayish-white *acholic* stool is formed.

Under normal conditions the odor of feces is due to skatole and indole, two bodies formed in the course of putrefactive processes occurring within the intestine (see page 294). Such bodies as methane, methyl mercaptan, and hydrogen sulphide may also add to the disagreeable character of the odor. The intensity of the odor depends to a large degree upon the character of the diet, being very marked in stools from a meat diet, much less marked in stools from a vegetable diet, and frequently hardly detectable in stools from a milk diet. Thus the stool of the infant is ordinarily nearly odorless and any decided odor may generally be readily traced to some pathological source.

Experiments in which the actual hydrogen ion concentration of the feces was determined indicate that the normal reaction of the excreta is slightly alkaline (pH 7.0 to 7.5).[2] Pronounced dietary changes, *e.g.*, low protein diet, high protein diet, fasting, water drinking with meals, produce at most only minor changes in the reaction of the feces. The ingestion of large amounts of lactose may cause the production of an acid reaction.

The form and consistency of the stool is dependent, in large measure, upon the nature of the diet. Under normal conditions the consistency may vary from a thin, pasty discharge to a firmly formed stool. Stools which are exceedingly thin and watery ordinarily have a pathological significance. In general the feces of the carnivorous animals are of a firmer consistency than those of the herbivora.

The continued ingestion of a diet which is very thoroughly digested and absorbed is frequently accompanied by the formation of dry, hard fecal masses (*scybala*). Constipation generally results, due to the small bulk of the feces and its lack of moisture. At present the formation of scybala is considered pathological, as an expression of spastic constipation. To counteract this tendency toward constipation the ingestion

[1] Quincke: *Münch. med. Woch.*, p. 854, 1896.

[2] Howe and Hawk: *Jour. Biol. Chem.*, 11, 129, 1912; Robinson: *Jour. Biol. Chem.* 52, 445, 1922; 66, 811, 1925.

of agar-agar[1] is practiced.[2] This agar is relatively indigestible and readily absorbs water (about 16 times its weight), thus forming a bulky fecal mass which is sufficiently soft to permit of easy evacuation. Agar is not limited to its use in connection with constipation; it may serve in other capacities as an aid to intestinal therapeutics by serving as a vehicle for certain drugs.[3] Mineral oil, because of its lubricating and softening properties is much used as an aid in overcoming constipation. It is inert and non-absorbable.

It is frequently desirable for clinical or experimental purposes to make an examination of the fecal output which constitutes the residual mass from a certain definite diet. Under such conditions, it is customary to cause the person under observation to ingest some substance, at the beginning and end of the period in question, which shall sufficiently differ in color and consistency from the surrounding feces as to render comparatively easy the differentiation of the feces of that period from the feces of the immediately preceding and succeeding periods. One of the most satisfactory methods of making this "separation" is by means of the ingestion of a gelatin capsule containing about 0.2 gram of powdered charcoal at the beginning and end of the period under observation. This procedure causes the appearance of *two black zones* of charcoal in the fecal mass and thus renders comparatively simple the differentiation of the feces of the intermediate period. Carmine (0.3 gram) may be used in a similar manner and forms two *dark red zones*. Some similar method for the "separation of feces" is universally practised in connection with the scientifically accurate type of nutrition or metabolism experiment which embraces the collection of useful data regarding the income and outgo of nitrogen and other elements.

Among the macroscopical constituents of the feces may be mentioned the following: Intestinal parasites and their ova, undigested food particles, gall stones, pathological products of the intestinal wall, enteroliths, intestinal sand, and objects which have been accidentally swallowed.

The fecal constituents which at various times and under different conditions may be detected by the use of the microscope are as follows: Constituents derived from the food, such as *muscle fibers*, *connective-tissue shreds*, *starch granules*, and *fat;* form elements derived from the intestinal tract, such as *epithelium*, *erythrocytes*, and *leucocytes; mucus; pus corpuscles; parasites* and *bacteria*. In addition to the constituents named the following *crystalline deposits* may be detected:

[1] Agar-agar is a product prepared from certain types of Asiatic sea-weed. It is a carbohydrate and is classified as a *galactan* in the polysaccharide group.

[2] Mendel: *Zent. f. ges. Physiol. u. Path. des Stoffw.*, No. 17, p. 1, 1908; Schmidt: *Münch. med. Woch.*, 52, 1970, 1905.

[3] Einhorn: *Berl. klin. Woch.*, 49, 113, 1912.

cholesterol, *koprosterol*, *soaps*, *fatty acid*, *fat*, *hematoidin*, "*triple phosphate*," *Charcot-Leyden crystals*, and the *oxalate*, *carbonate*, *phosphate*, *sulphate*, and *lactate* of calcium. (See Figs. 89 to 94, pp. 317 and 318.)

It is claimed by Sperry and Bloor[1] that the "fat" of feces does not arise from unabsorbed fatty material from the food. (See Chapter XXXII.)

The koprosterol of the feces is similar to cholesterol, and may be formed by the reduction of the latter. It responds to cholesterol color tests and has the same solubility, but possesses a lower melting-point and crystallizes in fine needles instead of plates such as cholesterol forms.

The detection of minute quantities of blood in the feces ("occult blood") has recently become a recognized aid to a correct diagnosis of certain disorders. In these instances the hemorrhage is ordinarily so slight that the identification by means of macroscopical characteristics as well as the microscopical identification through the detection of erythrocytes are both unsatisfactory in their results. Of the tests given for the detection of "occult blood" the benzidine reaction, Lyle-Curtman guaiac procedure and the ortho-tolidin tests (pp. 320–321) are probably the most satisfactory. Since "occult blood" occurs with considerable regularity and frequency in gastrointestinal cancer and in gastric and duodenal ulcer, its detection in the feces is of especial value as an aid to a correct diagnosis of these disorders. Certain precautions are essential, such as the establishment of a meat-free diet over a period of time before the specimen is collected. (Feces from a meat diet will give an occult blood reaction with some of the most delicate tests.) Bleeding from the bowel such as is seen in hemorrhoids, as well as the admixture of menstrual blood, is to be considered in the interpretation of the result.

FIG. 87.—CHARCOT-LEYDEN CRYSTALS.

It has been quite clearly shown that the intestine of the newly born is sterile. However, this condition is quickly altered and bacteria may be present in the feces before or after the first ingestion of food. There are three possible means of infecting the intestine, *i.e.*, by way of the mouth or anus or through the blood. The infection by means of the blood seldom occurs except under pathological conditions, thus limiting the general infection to the mouth and anus.

In infants with pronounced constipation two-thirds of the dry substance of the stools has been found to consist of bacteria. In the stools of normal adults probably about one-third of the dry substance is bacteria.[2] The average excretion of dry bacteria in 24 hours for an

[1] Sperry and Bloor: *Jour. Biol. Chem.*, 60, 261, 1924.

[2] Schittenhelm and Tollens found bacteria to comprise 42 per cent of the dry matter. This value is, however, undoubtedly too high.

adult is about 8 grams. The output of fecal bacteria has been found to undergo a decrease under the influence of water drinking with meals.[1] There was also a decrease in intestinal putrefaction,[2] a fact which indicates that at least a part of the bacterial deficit was made up of putrefactive organisms. In some cases over 50 per cent of the total nitrogen of feces has been shown to be *bacterial nitrogen*.[3]

Various enzymes have been detected in the feces. The first one so demonstrated was pancreatic amylase.[4] The amylase content of the feces has been considered to be an index of pancreatic activity.[5] The excretion of this enzyme has been found to increase under the influence of water drinking with meals.[6] Other enzymes which have been found in the feces under various conditions are trypsin, rennin, maltase, sucrase, lactase, nuclease and lipase.[7] In an abnormally rapid transit of food through the intestinal tract, such as is seen in certain diarrheas, nearly all of these enzymes may be detected.

Some of the more important organisms met with in the feces are the following:[8] *B. coli*, *B. lactis aërogenes*, *Bact. Welchii*, *B. bifidus*, and *coccal forms*. Of these the first three types mentioned are *gas-forming* organisms. The production of gas by the fecal flora in dextrose-bouillon is subject to great variations under pathological conditions; alterations in the diet of normal persons will also cause wide fluctuations. Data as to the production of gas are of considerable importance in a diagnostic way, although the exact cause of the variation is not yet established. It should be borne in mind in this connection that gas volumes are frequently variable with the same individual. For this reason it is necessary in every instance to follow the gas production for a considerable period of time before drawing conclusions.[8] While the question of the study of bacterial flora of the feces is a question beyond the range of this work, mention may be made here of the character of the organisms observed by Gram staining of the stool after administration of different types of diet. It has been shown that when the diet is markedly protein, the protein type of flora becomes predominant in the stools. Gram-stained smears show a fairly equal distribution of Gram-negative and Gram-positive organisms. Among the latter are largely the subtiloid organisms with some of the *Bact. Welchii*, together

[1] Mattill and Hawk: *Jour. Am. Chem. Soc.*, 33, 1999, 1911; Blatherwick and Hawk: *Bioch. Bull.*, 3, 28, 1913.
[2] Hattrem and Hawk: *Arch. Int. Med.*, 7, 610, 1911; Sherwin and Hawk: *Jour. Am. Chem. Soc.*, 36, 1779, 1914.
[3] MacNeal, Latzer and Kerr: *Jour. Inf. Dis.*, 6, 123, 1909; Mattill and Hawk: *Jour. Exp. Med.*, 14, 433, 1911; Blatherwick and Hawk: *Biochem. Bull.*, 3, 28, 1913.
[4] Wegscheider: *Inaug. Diss.*, Strassburg, 1875.
[5] Wohlgemuth: *Berl. klin. Woch.*, 47, 3, 92, 1910.
[6] Hawk: *Arch. Int. Med.*, 8, 382, 1911.
[7] Ury: *Biochem. Zeit.*, 23, 152, 1909.
[8] Herter and Kendall: *Jour. Biol. Chem.*, 5, 283, 1908.

with a moderate number of *diplococci* and *coccoid* forms. Most of the Gram-negative organisms resemble the *B. coli*. When the diet is carbohydrate the field is strongly Gram positive and has a more homogeneous appearance. The bacteria seen consist chiefly of long slender Gram-positive rods belonging to the *B. acidophilus* and *B. bifidus* groups.[1]

The nitrogen present in the feces exists principally in the form of *bacteria, unabsorbed intestinal secretions* and *digestive juices, epithelial cells, mucus material* and *food residues*. In the early days of nutrition study the fecal nitrogen was believed to consist principally of food residues. We now know that such residues ordinarily make up but a small part of the nitrogen quota of the stools of normal individuals who exercise normal mastication.[2] When meat has been "bolted," however, from 0.5 gram to 16 grams of macroscopical meat residues have been found in a single stool.[3] The phrase "metabolic product nitrogen" is frequently used as a designation for all fecal nitrogen except that present as food residues and bacteria. Bacteria cannot logically be classed under "metabolic" nitrogen since they doubtless develop at the expense of food nitrogen as well as at the expense of that in the form of intestinal secretions. In the accurate study of "protein utilization"[4] a correction should be made for "metabolic nitrogen." Data regarding the output of metabolic nitrogen may be secured by determining the fecal nitrogen excretion on a diet of proper energy value but *containing no nitrogen*.[5] Agar-agar may be utilized advantageously in connection with such a nitrogen-free diet.

The importance of the intestine as an excretory organ has been emphasized particularly by Myers and Fine[6] who have reported quite an extended study of the inorganic constituents of feces.

Feces are still excreted from the intestine even when no food is ingested. Carefully conducted fasting experiments have demonstrated this. A dog nourished on an ordinary diet to which *bone ash* has been added will excrete *grey feces*. When fasted such an animal will, after a few days, excrete a small amount of a greenish-brown mass, *containing no bone ash*. These are *fasting feces*. They are of a pitch-like consistency and turn black on contact with the air.[7] Adult fasting men have been

[1] Cammidge: The Feces of Children and Adults, 1914, p. 126.
[2] Kermauner: *Zeit. für Biol.*, 35, 316, 1897.
[3] Foster and Hawk: *Jour. Am. Chem. Soc.*, 37, 1347, 1915.
[4] The percentage of the ingested protein which is absorbed from the intestine. To calculate this factor subtract the metabolic nitrogen from the total fecal nitrogen and then subtract this value from the food nitrogen and divide by the food nitrogen. (See "Protein Utilization," Chapter XXXII.)
[5] Tsuboi: *Zeit. für Biol.*, 35, 68, 1897; Mendel and Fine: *Jour. Biol. Chem.*, 11, 5, 1912.
[6] Myers and Fine: *Proc. Soc. Exp. Biol. and Med.*, 16, 73, 1919.
[7] Howe and Hawk: *Jour. Am. Chem. Soc.*, 33, 215, 1911.

found to excrete 7–8 grams of feces per day, the daily nitrogen value being about 0.1 gram.[1] No separating medium such as charcoal or carmine (page 311) should be used in differentiating fasting feces.

In recent years the examination of feces for evidences of parasitism (detection of parasites and their ova) has taken on an added importance. The investigation of the hookworm has been particularly developed.

For diagnostic purposes the macroscopical and microscopical examinations of the feces ordinarily yield much more satisfactory data than are secured from its chemical examination. Possibly with the exception of certain examinations for occult blood, the most satisfactory data for diagnostic purposes are secured by microscopical examination. This presupposes a knowledge of microscopical technic and the use of certain microchemical tests, by which much information can be obtained. The principle underlying this examination consists in the study of the actual changes which the various food-stuffs have undergone during digestion. A knowledge of the changes which occur in normal digestion and which are seen in normal feces enables one to readily detect pathological variations. One diet widely used for this purpose is the Schmidt diet which is given below. The modification[2] described is better adapted to American conditions.

The *original Schmidt intestinal diet* is as follows:

In the *morning:* 0.5 liter of milk, or if milk does not agree 0.5 liter of cocoa (prepared from 20 grams of cocoa powder, 10 grams sugar, 400 grams water, and 100 grams milk). To this add 50 grams zwiebach.

In the *forenoon:* 0.5 liter oatmeal gruel (made from 40 grams oatmeal), 10 grams butter, 100 grams milk, 300 grams water, 1 egg strained.

At *noon:* 125 grams of chopped beef (raw weight) broiled rare with 20 grams of butter, so that the interior will remain raw. To this add 250 grams potato broth (made of 190 grams mashed potatoes, 100 grams milk, 10 grams of butter).

In the *afternoon:* as in the morning.

In the *evening:* as in the forenoon.

This diet necessitates five meals a day especially prepared and does not follow the average American dietary. In simple microscopical examinations for food digestion, the following diet as more closely approximating the ordinary dietary regime is suggested. Should chemical determinations for fat be desired all fat containing foods can be eliminated except those in which its specific content is known and a measured amount of fat given. The feces can then be separated by means of carmine.

[1] Howe, Mattill and Hawk: *Ibid.*, 33, 568, 1911.
[2] Used by Dr. Rehfuss of Philadelphia.

Modified Schmidt Diet

Breakfast:
100 grams cream of wheat or oatmeal
60 grams toast
20 grams butter
250 c.c. milk.

Luncheon:
Rice soup (chicken broth with rice)
100 grams green vegetable (asparagus)
100 grams mashed potato
60 grams toast
20 grams butter
250 c.c. milk.

4 o'clock:
250 c.c. of milk.

Dinner:
150 grams of chopped meat, grilled on the outside and rare in the center
100 grams green vegetable (spinach)
100 grams mashed potatoes
60 grams of toast
20 grams of butter
250 c.c. milk
Stewed fruit.

Experiments on Feces

1. Macroscopical Examination.—If the stool is watery pour it into a shallow dish and examine directly. If it is firm or pasty it should be treated with water and carefully stirred before the examination for macroscopical constituents is attempted. The macroscopical constituents may be collected very satisfactorily by means of a double layer of cheese cloth.

A Boas sieve (Fig. 88) may also be used to collect the macroscopical constituents of feces. This sieve is constructed of two easily detachable hemispheres which are held together by means of a bayonet catch. In using the apparatus the feces are spread out upon a very fine sieve contained in the lower hemisphere and a stream of water is allowed to play upon it through the medium of an opening in the upper hemisphere. The apparatus is provided with an orifice in the upper hemisphere through which the feces may be stirred by means of a glass rod during the washing process. After 15–30 minutes washing nothing but the coarse fecal constituents remain upon the sieve.

Fig. 88.—Boas Sieve.

2. Microscopical Examination.—After the ingestion of the test diet (see Schmidt diet above) for several days, a specimen of the movement is collected. Any gross abnormalities are recorded in the form, consistence, and character of the stool as well as the admixture of certain pathological elements such as pus, blood, mucus, and parasites. The movement is then rubbed out on plates and the presence of undigested food-stuffs sought for. Normally the test diet is almost completely digested and no gross undigested material is found. Therefore the presence of these macroscopic rests is in itself evidence

of disturbed digestion. Clean slides and cover-glasses are then prepared and a small representative portion of the movement is placed on each of three slides. The routine clinical method of examination follows: To the first slide is added a drop of distilled water and it is then examined with low and high powers.

FIG. 89.—*A*, intact undigested meat fibers; *B*, partially digested meat fibers; *C*, almost completely digested meat fibers.

FIG. 90.—*A*, neutral fat; *B*, fatty acid liberated by acetic acid; *C*, soaps; *D*, fatty acid crystals.

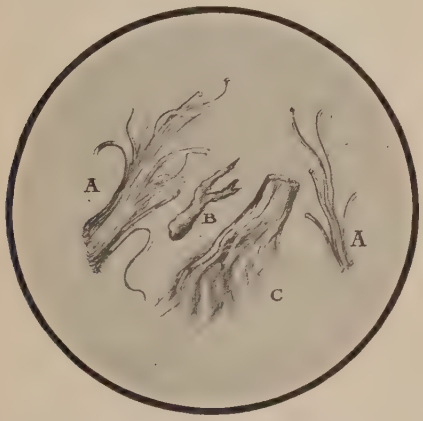


FIG. 91.—*A*, elastic tissue; *B*, white fibrous tissue (macroscopic); *C*, white fibrous tissue (microscopic.)

FIG. 92.—*A*, cellulose remains of vegetables; *B*, empty potato cells; *C*, potato cells filled with starch, and stained with iodine; *D*, hard cells found in pears; *E*, spiral and woody fibers from pith of vegetables; *F*, vegetable hairs.

FIGS. 89 TO 92.—MICROSCOPICAL CONSTITUENTS OF FECES.

Meat fibers are readily recognized by their yellowish hyaline appearance possibly with a few striæ still visible in the fibers. Should meat fibers be found bound together by connective tissue or raw connective tissue, either white fibrous or yellow elastic, be noted, it

indicates a disturbance of gastric function inasmuch as one of the specific functions of the gastric juice is to dissolve the intercellular tissue binding together the fibers. If large numbers of meat fibers are found after a test diet, particularly if the nuclei are still intact in the fibers, the inference of poor or low pancreatic function is justifiable. This is true if it can be demonstrated that the food has been sufficiently long in its transit through the intestinal tract to permit the pancreatic enzymes to carry on their work. A dilute solution of methylene blue will readily show the nuclei if present.

The second slide is examined for fats and then treated with acetic acid and heated to split any soaps which may be present and form fatty acid.

Fats are met with in three forms (a) neutral fats readily demonstrated by Sudan III, Scharlach R or Osmic acid; (b) fatty acids which are usually found in the form of needle-like crystals soluble in ether, alcohol, and solutions of sodium

FIG. 93.—*A*, calcium sulphate crystals; *B*, cholesterol crystals; *C*, charcoal detritus; *D*, bismuth sub-oxide crystals; *E*, calcium oxalate crystals.

FIG. 94.—*A*, Schmidt test bag for study of pancreatic function; *B*, nuclei of meat fibers digested; *C*, nuclei of meat fibers undigested; *D*, undigested stained thymus cells.

FIGS. 93 AND 94.—MICROSCOPICAL CONSTITUENTS OF FECES.

hydrate (these crystals do not stain with Sudan III but form drops on being warmed); (c) soaps are usually found in the feces either as amorphous flakes or scallop shell-like formations, but may occasionally occur in crystalline form. The calcium soaps which compose the bulk of the soaps in the feces can be distinguished from the potassium and sodium compounds because of their insolubility in hot water, alcohol, and ether. On heating with 30 per cent acetic acid, fatty acids are set free in drops which crystallize out on cooling.

The estimation of fats is a rather important matter and the trained observer can usually detect disturbances in fat digestion. Normally there are fats present in the movement, but abnormally their quantity is relatively increased either in total fat, or in one of its components.

While it is true that bacterial activity plays a considerable rôle in the digestion of fats, a marked increase in fat usually indicates pancreatic disease, or a disturbance in pancreatic function. This is, of course, the case only when the amount of fat ingested is not in excess of that which can be readily handled under normal conditions. In cases of pure biliary obstruction without pancreatic involvement, fat-splitting takes place in a normal way, but the fatty acids and soaps formed are not absorbed owing to the absence of bile. Such a movement is full of soaps and fatty acid crystals which on treatment with acetic acid show a marked increase in total fat over normal. Failure of absorption owing to extensive disease of the intestinal mucosa can produce a similar picture but will usually give some cytological evidence of intestinal disease. Pure pancreatic disease gives a marked increase in total and neutral fat with the presence of bile.

Undigested starches are readily recognized by their blue reaction with iodine. This can be studied on the third slide.

This phenomenon is the least frequent among the different forms of pathological digestion and usually indicates food bolting, an excessive ingestion of, or poor preparation of carbohydrate food, or an infection of the bowel with so-called "gärungsdyspepsia" rather than an actual disturbance of pancreatic function inasmuch as the amylolytic function of the pancreas is the most persistent and the last to disappear.

Disturbance in cellulose digestion, the presence of blood, leucocytes, mucus, etc., can all be demonstrated by appropriate technic and represent a chapter in the study of the feces of great diagnostic importance, but one which is beyond the province of this volume. (For further discussion, see page 311. For cuts of fecal constituents found microscopically, see pages 317 and 318.)

3. Reaction.—Thoroughly mix the feces and apply moist red and blue litmus papers to the surface. If the stool is hard it should be mixed with water before the reaction is taken. Examine the stool as soon after defecation as is convenient, since the reaction may change very rapidly. The reaction of the normal stools of adult man is ordinarily neutral or faintly alkaline to litmus, but seldom acid. Infants' stools are generally acid in reaction. Try the reaction to Congo red paper. Also test the reaction of fecal extract to phenolphthalein.

4. Starch.—If any imperfectly cooked starch-containing food has been ingested it will be possible to detect starch granules by a microscopical examination of the feces. If the granules are not detected by a microscopical examination, the feces should be placed in an evaporating dish or casserole and boiled with water for a few minutes. Filter and test the filtrate by the iodine test in the usual way (see page 93).

5. Cholesterol, Koprosterol and Fat.—Introduce about 5 grams of moist feces into a 100 c.c. glass-stoppered cylinder. Add 30 c.c. of distilled water and 25 c.c. of ether, then stopper the cylinder and shake vigorously for five minutes.

Allow to separate, pour or pipette off the ethereal solution. Filter and remove the ether by evaporation. The residue contains cholesterol and the mixed fats of the feces. For every gram of fat add about 1.5 grams of solid potassium hydroxide and 25 c.c. of 95 per cent alcohol and boil in a flask on a water-bath for one-half hour, maintaining the volume of alcohol constant. This alcoholic-potash has saponified the mixed fats and we now have a mixture of soaps, cholesterol and koprosterol. Add sodium chloride, in substance, to the mixture and extract with ether to dissolve out the cholesterol and koprosterol. Remove the ether by evaporation and examine the residue microscopically for cholesterol and koprosterol crystals. Try any of the other tests for cholesterol as given on page 281.

6. Blood.—Undecomposed blood may be detected macroscopically. If uncertain, look for erythrocytes under the microscope, and spectroscopically for the spectrum of oxyhemoglobin (see Absorption Spectra, Plate I).

In case the blood has been altered or is present in minute amount ("occult blood"), and cannot be detected by the means just mentioned, the following tests may be tried:

(a) Benzidine Reaction.—Make a thin fecal suspension using about 5 c.c. of distilled water. Shake with 5 c.c. of ether to remove fat and discard this ether extract. Acidify the residue with acetic acid and extract again with 5 c.c. of ether. Pour the acid ether extract into a small evaporating dish. Evaporate to dryness on a hot water bath (with flame turned out). Add a few drops of water a drop of saturated solution of benzidine in glacial acetic acid and a drop of 3 per cent hydrogen peroxide. A blue or green color indicates the presence of blood.

Wagner[1] has simplified the benzidin test so that it can be applied much more conveniently.

Slide Modification.—Take up a little of the solid stool on a match, smear it on an object glass and pour the reagent over it. It turns blue if there is blood present and there is no misleading green tint from fluid. Make the solution as follows: Add a knife-tip of benzidine to 2 c.c. of glacial acetic acid, and add 20 drops of a 3 per cent solution of hydrogen peroxide.

By this dry technic there is no danger of soiling the fingers, and the test is more sensitive than the usual "wet" benzidine test. The smear of stool is either blue or it is not blue. The rapidity of the color change gives some idea as to the proportion of blood in the stool; with much blood present the change to blue is instantaneous. It is claimed by Vaughn[2] that pus and the usual drugs and foods ingested do not interfere with the reaction.

(b) Lyle-Curtman Guaiac Procedure.[3]—Approximately 10 gm. of the stool are transferred to a beaker, 25 c.c. of distilled water are added, and the mixture is stirred until of uniform consistency. Over a low flame, the mixture is heated

[1] Wagner: *Zentbl. für Chirurgie*, 41, No. 28, 1914.
[2] Vaughn: *Jour. of Lab., Clin. Med.*, 2, 437, 1917.
[3] Lyle and Curtman: *Jour. Biol. Chem.*, 33, 1, 1918.

with constant stirring to boiling and kept at the boiling temperature for several minutes. After cooling, one-half of the mixture is transferred to a glass-stoppered bottle of 80 c.c. capacity, 5 c.c. of glacial acetic acid and 25 c.c. of ether are added, and the mixture is thoroughly shaken and allowed to stand for several minutes. In a test tube, 2 c.c. of the ether extract are treated with 0.5 c.c. (1:60) of the Lyle-Curtman guaiac reagent[1] and finally one to five drops of 30 per cent perhydrol are added slowly from a pipette. A decided green, light or dark blue, or purple color indicates the presence of blood in quantity to be of clinical significance.

(c) Ortho-tolidin Test (Ruttan and Hardisty).[2]—To 1 c.c. of a 4 per cent glacial acetic acid solution of o-tolidin[3] in a test-tube add 1 c.c. of the solution under examination and 1 c.c. of 3 per cent hydrogen peroxide. In the presence of blood a bluish color develops (sometimes rather slowly) and persists for some time (several hours in some instances).

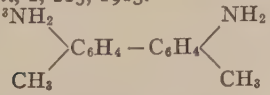
This test is said to be as sensitive for the detection of occult blood in feces and stomach contents as is the benzidine reaction. It is also claimed to be more satisfactory for urine than any other blood test. The acetic acid solution may be kept for one month with no reduction in delicacy.

(*d*) *Phenolphthalein Test.*[4]—Make a thin fecal suspension using about 5 c.c. of distilled water. Heat to boiling,[5] cool and add 2 c.c. of the suspension to 1 c.c. of

[1] The Lyle-Curtman Guaiac Reagent.—Fifty gm. of the ground crude gum guaiac are treated in a beaker with 20 gm. of KOH dissolved in 200 c.c. of water. After thorough stirring, the mixture is filtered with the aid of suction through cotton spread out in a thin layer in a Buchner funnel. The residue is washed with water until the combined filtrate and washings approximate 1.5 liters. To the diluted KOH solution are added with constant stirring 21 c.c. of glacial acetic acid which is run dropwise from a burette. The precipitate is allowed to settle, the supernatant liquid poured off, and the residue washed once with water by decantation. The precipitate is then transferred to a Buchner funnel and dried by suction as much as possible. The precipitate is gently heated (small portions at a time) in an evaporating dish when most of the water separates and is removed by filter paper. After the removal of the water, and while the mass is still plastic, it is drawn out into thin sheets. In this condition the material rapidly hardens and dries in the air. The dried masses are then ground, treated with 300 c.c. of hot 95 per cent alcohol and the mixture is thoroughly stirred to prevent the formation of a gummy mass. In a few minutes a dark brown material separates in a flocculent condition. This is filtered off and the alcohol removed from the solution by distillation. The residue in the flask is treated with 20 gm. of KOH dissolved in water, diluted considerably, and precipitated as before with about 20 c.c. of glacial acetic acid. The precipitate is filtered off and dried as described above, after which it is ground and kept in a desiccator. The weight of the material finally obtained represents a yield of about 60 per cent. The time required to make this preparation is 4 hours, the distillation of the alcohol being the most time-consuming of all the operations.

A solution containing 1 gm. of this preparation in 60 c.c. of 95 per cent alcohol may be prepared and kept in a glass-stoppered bottle of colorless glass. This reagent does not deteriorate for several weeks.

[2] Ruttan and Hardisty: *Canadian Medical Ass'n. Journal*, Nov., 1912, also *Biochemical Bull.*, 2, 225, 1913.

[3] 

[4] Boas: *Deut. med. Woch.*, 37, 62, 1911.

[5] Boas suggests using an ether extract of the fecal suspension thus eliminating the necessity of boiling. However, oxidizing enzymes are the main sources of error here and the action is easily and effectively eliminated by boiling. (See White: *Boston Medical and Surgical Journal*, 164, 876, 1911.)

the phenolphthalein reagent[1] and a few drops of hydrogen peroxide. *A pink* or *red* color promptly forms in the presence of blood.

Schirokauer[2] makes the statement that a mixture of alcohol and glacial acetic acid will give the phenolphthalein reaction for occult blood. The action of an oxidizing agent will make this reaction more distinct. Von Czylharz and Neustadl[3] find that a solution of sodium salicylate added to a blood-free extract of feces will give a very deceptive reaction, while feces after the administration of sodium salicylate by mouth gave the same reaction. The same was true of acetyl salicylic acid and other similar drugs. Their studies in clinical cases likewise indicated that the phenolphthalein test was unreliable.

(e) **Cowie's Guaiac Test.—To 1 gram of moist feces add 4–5 c.c. of glacial acetic acid and extract the mixture with 30 c.c. of ether. To 1–2 c.c. of the extract add an equal volume of water, agitate the mixture, introduce a few granules of powdered guaiac resin, and after bringing the resin into solution, gradually add 30 drops of old turpentine or hydrogen peroxide. A blue color indicates the presence of blood. Cowie claims that by means of this test an intestinal hemorrhage of 1 gram can easily be detected by an examination of the feces.**

7. Hydrobilirubin. Schmidt's Test.—Rub up a small amount of feces in a mortar with a concentrated aqueous solution of mercuric chloride. Transfer to a shallow, flat-bottomed dish and allow to stand 6–24 hours. The presence of hydrobilirubin will be indicated by a deep red color being imparted to the particles of feces containing this pigment. This red color is due to the formation of hydrobilirubin-mercury. If unaltered bilirubin is present in any portion of the feces that portion will be green in color due to the oxidation of bilirubin to biliverdin.

Another method for the detection of hydrobilirubin is the following: Treat the dry feces with absolute alcohol acidified with sulphuric acid and shake thoroughly. The acidified alcohol extracts the pigment and assumes a reddish color. Examine a little of this fluid spectroscopically and note the typical spectrum of hydrobilirubin (Absorption Spectra, Plate II).

8. Bilirubin.[4] (a) Gmelin's Test.—Place a few drops of concentrated nitric acid in an evaporating dish or on a porcelain test-tablet and allow a few drops of the feces and water to mix with it. The usual play of colors of Gmelin's test is produced, i.e., green, blue, violet, red, and yellow. If so desired, this test may be executed on a slide and observed under a microscope.

(*b*) *Huppert's Test.*—Treat the feces with water to form a semi-fluid mass, add an equal amount of milk of lime, shake thoroughly, and filter. Wash the precipitate with water, then transfer both the paper and the precipitate to a small beaker or flask, add a small amount of 95 per cent alcohol acidified slightly with sulphuric acid, and heat to boiling on a water-bath. The presence of bilirubin is indicated by the alcohol assuming a green color.

[1] Prepared by dissolving 1–2 grams of phenolphthalein and 25 grams of KOH in 100 c.c. of distilled water. Add 1 gram of powdered zinc and heat gently until the solution is decolorized. Prepared in this way the solution will not deteriorate on standing.

[2] *Deutsch. med. Woch.*, Aug. 6, 1914.

[3] *Wien. med. Woch.*, Sept. 5, 1914.

[4] The detection of bilirubin in the feces is comparatively simple provided it is not accompanied by other pigments. When other pigments are present, however, it is difficult to detect the bilirubin and, at times, may be found impossible.

Steensma advises the addition of a drop of a 0.5 per cent solution of sodium nitrite to the acid-alcohol mixture before warming on the water-bath. Try this modification also.

9. Bile Acids.—Extract a small amount of feces with alcohol and filter. Evaporate the filtrate on a water-bath to drive off the alcohol and dissolve the residue in water made slightly alkaline with potassium hydroxide. Upon this aqueous solution try any of the tests for bile acids given on page 279.

10. **Inorganic Constituents.**—Incinerate a small amount of feces in a crucible and dissolve the ash in a small volume of dilute nitric acid. Dilute with water and filter. Make the following tests upon the clear filtrate.

(*a*) *Chlorides.*—Acidify with nitric acid and add silver nitrate.

(*b*) *Phosphates.*—Acidify with nitric acid, add molybdate solution, and warm gently.

(*c*) *Sulphates.*—Acidify with hydrochloric acid, add barium chloride, and warm.

(*d*) *Calcium.*—Neutralize with ammonium hydroxide, make slightly acid with acetic acid and add ammonium oxalate. Let stand.

(*e*) *Magnesium.*—Neutralize with ammonium hydroxide, and add Na_2HPO_4 and excess of NH_4OH. Let stand.

11. **Indole Reactions.—Rub up the stool with water to form a thin paste and distill first in alkaline and then in acid solution. Test the distillate by any of the tests for the detection of indole in putrefaction mixtures (see page 303).**

12. **Schmidt's Nuclei Test.**—This test serves as an aid to the diagnosis of pancreatic insufficiency. The test is founded upon the theory that cell nuclei are digestible *only* in pancreatic juice, and therefore that the appearance in the feces of such nuclei indicates insufficiency of pancreatic secretion.

The procedure is as follows: Cubes of fresh beef about 0.5 cm. square are enclosed in small gauze bags and ingested with a test meal. Subsequently the fecal mass resulting from this test meal is examined, the bag opened, and the condition of the enclosed residue determined. Under normal conditions the nuclei would be digested. Therefore if the nuclei are found to be for the most part undigested, and the intervening period has been sufficient to permit of the full activity of the pancreatic function (at least six hours), it may be considered a sign of pancreatic insufficiency (see Fig. 94, p. 318).

It has been claimed by Steele that under certain conditions the non-digestion of the nuclei may indicate a general lowering of the digestive power rather than a true pancreatic insufficiency.

Kashiwado[1] has suggested the use of stained cell nuclei in this test. A preparation put out under the name "Gefarbte gewebskerne zur Pankreasfuntionsprüfung nach Prof. Dr. Schmidt und Dr. Kashiwado" consists of a mixed preparation of thymus cells, the nuclei of which are stained by iron hematoxylin, and lycopodium powder. After

[1] Kashiwado: *Deut. Arch. Klin. Med.*, 104, 584, 1911.

administration, the lycopodium, which is readily recognized, is sought for in the stool and when found that portion is examined for the stained thymus cells. Their statement is as follows: If stained nuclei are not found in the feces after an intestinal transit of sufficient duration (at least six hours) normal pancreatic function (external) is indicated. If, however, all or part of the cells are found, a definite disturbance in pancreatic function is present.

13. Influence of Drugs upon the Color of the Stool.—Ingest an ordinary mixed diet, take the indicated dose of one of the following drugs, "separate" the feces (see page 325) and after the "marker" appears note the color of the stools evacuated:

Drug	Dose	Color of stool
Bismuth subnitrate, grams.	5	Black.
Calomel, mg.....	130–140	Green.
Reduced iron, mg.....	65–70	Grayish black turning darker on exposure to air.
Methylene blue, mg.....	130–140	Blue, especially after exposure to air.
Manganese dioxide, mg...	130–140	Dark brown or black.
Hematoxylin, grams.....	1	Reddish brown.
Rhubarb, c.c. fluid extract	2	Yellow.
Senna, c.c. fluid extract...	4	Dark yellow.
Cambogia, mg.....	130–140	Dark yellow.
Santonin, mg.....	65–70	Dark yellow.

14. Einhorn's Bead Test.[1]—This is a method for testing the digestive function. In some respects it is similar to Sahli's desmoid reaction (see Gastric Analysis). The procedure consists in wrapping the material under examination (catgut, fish-bone, raw beef, cooked potatoes, thymus gland or mutton fat, etc.) in gauze to which glass beads of various colors are attached and enclosing gauze and beads in a gelatin capsule.[2] The gelatin capsule is swallowed and the beads serve to facilitate the separation of the gauze from the feces. The residue within the gauze is then examined. If beads appear in much less than 24 hours

[1] Einhorn: *The Post-Graduate*, May, 1912: *Boas' Arch.*, 12, 26, 1906; 13, 35, 1907; *Ibid.*, 475; 15, part 2, 1909.

[2] Ordinarily two substances are attached to each bead, three beads tied together and enclosed in one capsule. Test capsules may be obtained from Eimer and Amend, New York.

an accelerated motility is indicated, whereas an interval of 48 hours or over elapsing indicates retarded motility. If gastric function alone is to be studied silk threads are attached to the beads and the latter are withdrawn and examined before they have passed into the intestine.

15. "Separation" of Feces.—In order to become familiar with the method ordinarily utilized in metabolism experiments to differentiate the feces which correspond to the food ingested during any given interval, and at the same time to secure data as to the length of time necessary for ingested substances to pass through the alimentary tract proceed as follows: Just before one of the three meals of the day ingest a gelatine capsule (No. 00) containing 0.2–0.3 of a gram of carmine or charcoal. Make an inspection of all stools subsequently dropped and note the time interval elapsing between the ingestion of the capsule and the appearance of its contents in the feces. Under normal conditions this period is ordinarily 24 hours. This test is thus an index of intestinal motility.

16. Influence of Foods upon the Color of the Stool.—Ingest a diet which contains a liberal quantity of one of the following articles of diet, "separate" the feces (see above) and after the "marker" appears note the color of the stools evacuated:

Article of diet	Color of stool
Milk	Light yellow or grayish white.
Meat	Brownish black.
Chlorophyllic vegetables, *e.g.*, spinach	Greenish.
Non-chlorophyllic vegetables	Light brown.
Cherries or blackberries	Reddish brown.
Cocoa	Dark red or chocolate brown.
Coffee	Dark brown.
Corn meal	Light colored.

17. Quantitative Determination of Fecal Amylase (The Author's[1] Modification of Wohlgemuth's[2] Method).—Weigh accurately about 2 grams of fresh feces into a mortar,[3] add 8 c.c. of a phosphate-chloride solution (0.1 mol dihydrogen sodium phosphate and 0.2 mol disodium hydrogen phosphate per liter of 1 per cent sodium chloride), 2 c.c. at a time, rubbing the feces mixture to a homogeneous consistency after each addition of the extraction medium. Permit the mixture to stand at room temperature for a half-hour with frequent stirring. We now have a neutral fecal suspension. Transfer this suspension to a 15 c.c. graduated centrifuge tube, being sure to wash the mortar and pestle carefully with the phosphate-chloride

[1] Hawk: *Arch. Int. Med.*, 8, 552, 1911.
[2] Wohlgemuth: *Berl. klin. Woch.*, 47, 3, 92, 1910; also see page 263.
[3] Duplicate determinations should be made.

solution and add all washings to the suspension in the centrifuge tube. The suspension is now made up to the 15 c.c. mark with the phosphate-chloride solution and centrifugated for a 15-minute period, or longer if necessary, to secure satisfactory sedimentation. At this point, read and record the height of the sediment column. Remove the supernatant liquid by means of a bent pipette, transfer it to a 50 c.c. volumetric flask and dilute it to the 50 c.c. mark with the phosphate-chloride solution. Mix the fecal extract thoroughly by shaking and determine its amylolytic activity. For this purpose a series of six graduated tubes is prepared, containing volumes of the extract ranging from 2.5 c.c. to 0.078 c.c. Each of the intermediate tubes in this series will thus contain one-half as much fluid as the preceding tube. Now make the contents of each tube 2.5 c.c. by means of the phosphate-chloride solution in order to secure a uniform electrolyte concentration. Introduce 5 c.c. of a 1 per cent soluble starch solution[1] and three drops of toluene into each tube, thoroughly mix the contents by shaking, close the tubes by means of stoppers and place them in an incubator at 38°C. for 24 hours. At the end of this time remove the tubes, fill each to within half an inch of the top with ice-water, add 1 drop of tenth-normal iodin solution, thoroughly mix the contents and examine the tubes carefully with the aid of a strong light. Select the last tube in the series which shows entire absence of blue color, thus indicating that the starch has been completely transformed into dextrin and sugar, and calculate the amylolytic activity on the basis of this dilution. In case of indecision between two tubes, add an extra drop of the iodin solution and observe them again.[2]

The amylolytic value, Df, of a given stool, may be expressed in terms of 1 c.c. of the sediment obtained by centrifugation as above described. For example, if it is found that 0.31 c.c. of the phosphate-chloride extract of the stool acting at 38°C. for 24 hours completely transformed the starch in 5 c.c. of a 1 per cent starch solution, then we would have the following proportion:

$$0.31 : 5 \text{ (c.c. starch)} :: 1 \text{ (c.c. extract)} : X$$

The value of X in this case is 16.1, which means that 1 c.c. of the fecal extract possesses the power of completely digesting 16.1 c.c. of a 1 per cent starch solution in 24 hours at 38°C.

[1] In preparing the 1 per cent solution, the weighed starch powder should be dissolved in cold distilled water in a casserole and stirred until a homogeneous suspension is obtained. The mixture should then be heated with constant stirring, until it is clear. This ordinarily takes from eight to ten minutes. A slightly opaque solution is thus obtained, which should be cooled and made up to the proper volume before using.

[2] Theoretically we would expect the colors to range from a light yellow to a dark blue, with red tubes holding an intermediate position in the series. This color sequence does often occur, but its occurrence is far from universal. Many times the first tubes in the series, *i.e.*, those containing the largest quantities of the fecal extract, will exhibit a bluish cast of color which should not be confused with the starch color reaction. When these blue tubes are present, they are generally followed by yellow, red and blue tubes in order, the final blue tube, of course, being the regulation starch reaction. Occasionally greenish colors will be obtained to the left of the red color. It also sometimes happens that it is somewhat difficult to determine in which tube to the right of the red color the starch blue color is first detected, unless the tube be examined carefully before a strong light. In every instance, however, when these blue and green colors are observed, it is noted that tubes possessing the true dextrin red color are always present between these tubes and the tubes possessing the true starch blue color. It is evident, therefore, that these bluish tints in the tubes to the left of the dextrin color cannot be due to the presence of starch. The cause of the blue color reaction in the first tubes of the series has not been ascertained as yet.

Inasmuch as stools vary so greatly as to water content, it is essential to an accurate comparison of stools that such comparison be made on the basis of the solid matter. Supposing, for example, that in the above determination we had 6.2 c.c. of sediment. Since the supernatant fluid was removed and made up to 50 c.c. before testing its amylolytic value, it is evident that 1 c.c. of this sediment is equivalent to 8.1 c.c. of extract. Therefore, in order to derive the amylolytic value of 1 c.c. of sediment, we must multiply the value (16.1) as obtained above for the extract, by 8.1. This yields 130.4 and enables us to express the activity as follows:

$$\mathrm{Df}\,{}^{38^\circ}_{24\mathrm{h}} = 130.4$$

The above method of calculation is that suggested by Wohlgemuth. In case time and facilities permit of the determination of the moisture content of the feces, it is much more accurate and satisfactory to place the amylolytic values of the stools on a "gram of dry matter" basis. The amylolytic values of the stools are expressed as the number of cubic centimeters of 1 per cent starch solution which the amylase content of 1 gram of dry feces is capable of digesting.

18. **Quantitative Determination of Fecal Bacteria.**[1]—The method is a simplification of MacNeal's adaptation of the Strasburger procedure.[2] About 2 grams of feces are accurately weighed and placed in a 50 c.c. centrifuge tube. To the feces in the tube a few drops of 0.2 per cent hydrochloric acid are added, and the material is mixed to a smooth paste by means of a glass rod. Further amounts of the acid are added with continued crushing and stirring until the material is thoroughly suspended. The tube is then whirled in the centrifuge at high speed for one-half to one minute. The suspension is found sedimented into more or less definite layers, the uppermost of which is fairly free from the larger particles. The upper and more liquid portion of the suspension is now drawn off by means of a pipette and transferred to a beaker.[3] The sediment remaining in the tube is again rubbed up with the glass rod with the addition of further amounts of dilute acid, and again centrifugalized for one-half to one minute. The supernatant liquid is pipetted off and added to the first, the same pipette being used for the one determination throughout.[4] A third portion of the dilute acid is then added to the sediment, which is again mixed by stirring and again centrifugalized. All the washings are added to the first one, and during the process care is taken to wash the material from the walls and mouth of the centrifuge tube down into it. Finally, when the sediment is sufficiently free from bacteria, the various remaining particles are visibly clean, and the supernatant liquid after centrifugalization remains almost clear. This is removed to the beaker in which are now practically all the bacteria present in the original portion of feces, together with some solid matter not yet separated. In the centrifuge tubes there is a considerable amount of bacteria-free solid matter.

The suspension is now transferred to the same centrifuge tube, centrifugalized for a minute, and the supernatant liquid transferred to a clean beaker by means of the same pipette. The tube is then refilled from the first beaker and thus all the suspension centrifugalized a second time. The beaker is finally carefully washed

[1] Mattill and Hawk: *Jour. Exp. Med.*, 14, 433, 1911.

[2] MacNeal, Latzer and Kerr, *Jour. Inf. Dis.*, 6, 123, 1909.

[3] A 25 c.c. pipette is the most satisfactory size; to facilitate observation, the delivery tube is bent near the bulb to an angle of about 120 degrees.

[4] A convenient support for the pipettes is a wire spring on a glass base, such as is used on a desk for pen-holders. The delivery tube, just where it is bent, is inserted between the wires, and any liquid not delivered collects in the bend of the tube.

with the aid of a rubber-tipped glass rod, the second sediment in the centrifuge tube is washed free of bacteria by means of this wash water and by successive portions of the dilute acid, and the supernatant liquid after centrifugalization is added to the contents of the second beaker. The second clean sediment is added to the first. The bacterial suspension now in the second beaker is again centrifugalized in the same way and a third portion of bacteria-free sediment is separated. Frequently a fourth serial centrifugalization is performed—always if the third sediment is of appreciable quantity. At all stages of the separation, small portions of the dilute hydrochloric acid are used, so that the final suspension shall not be too voluminous. Ordinarily it amounts to 125 to 200 c.c. At the same time, the final amount of fluid should not be too small, as shown by Ehrenpfordt,[1] because the viscosity accompanying increased concentration prevents proper and complete sedimentation.

To the final bacterial suspension an equal volume of alcohol is added and the beaker set aside to concentrate. A water-bath at 50° to 60°C. is very satisfactory. After two or three days, when the liquid is concentrated to about 50 c.c., the beaker is removed and about 200 c.c. of alcohol are added. The beaker is covered and allowed to stand at room temperature for 24 hours. At the end of this time the bacterial substance is generally settled, so that most of the clear supernatant liquid, of dark brown color, can be directly siphoned off without loss of solid matter. The remainder is then transferred to centrifuge tubes, centrifugalized, and the remaining clear liquid pipetted off.[2] The sediment consists of the bodies of the bacteria, and is transferred to a Kjeldahl flask for nitrogen determination. This is the bacterial nitrogen. Where a determination of bacterial dry substance is desired, the sediment of bacteria is extracted by absolute alcohol and ether in succession, transferred to a weighed porcelain crucible, and dried at 102°C. to constant weight. This dried sample is then used in the nitrogen determination. This procedure differs from that of MacNeal in that the bacterial dry matter is not determined. A saving of about seven days' time and of considerable labor is accomplished by this omission.

Inasmuch as it has been shown by various investigators that such bacteria as are present in the feces contain on the average about 11 per cent of nitrogen, the values for bacterial nitrogen as determined by our method may conveniently serve as a basis for the calculation of the actual output of bacterial substance.

19. Quantitative Determination of Indol in Feces. Bergeim's Modification of the Herter-Foster Method.[3]—*Principle.*—The feces are distilled from alkaline solution to remove phenols. This distillate is again distilled from acid solution to remove ammonia. The indol in the final distillate is treated with β-naphthaquinone sodium monosulphonate and alkali and the blue compound formed extracted with chloroform and determined colorimetrically.

Procedure.—Rub 30–50 grams of the fresh, well-mixed feces in a mortar with water to a uniform consistency. Transfer to a wide mouth Kjeldahl flask of about 1000 c.c. capacity, rinsing mortar and neck of flask with distilled water to make about 400 c.c. Add 5 c.c. of 10 per cent KOH solution and about 2 c.c. of paraffin to decrease foaming. Distill with steam using ordinary Kjeldahl distillation ap-

[1] Ehrenpfordt: *Zeit. exp. Path. Ther.*, 7, 455, 1909.

[2] In later work (see Blatherwick and Hawk: *Biochem. Bull.*, 3, 28, 1913) it was found advantageous to centrifugalize with alcohol and ether in succession before transferring the bacterial cells to Kjeldahl flasks.

[3] Herter and Foster: *Jour. Biol. Chem.*, 1, 257, 1906. Bergeim: *Jour. Biol. Chem.* 32, 17, 1917.

paratus with good stream of water in the condenser. Heat carefully for a few minutes until danger of foaming is past and then allow to boil vigorously. Distill over 500 c.c. of liquid, bringing the volume of the fecal suspension down to about 100 c.c. toward the end of the distillation.

Transfer the distillate to a clean Kjeldahl flask, add 2 drops of phenolphthalein as an indicator. Make neutral with N sulphuric acid and add 1 c.c. excess. Distill with steam as before, collecting the first 500 c.c. of distillate and bringing the residue finally to about 100 c.c. Mix distillate well by shaking.

Take an aliquot portion of the distillate (100 c.c); add 1 c.c. of a 2 per cent solution of β-naphthaquinone sodium monosulphonate solution. Then add 2 c.c. of 10 per cent KOH. Shake and let stand for 15 minutes. This is best carried out in a 150 c.c. Squibb shape separatory funnel. Extract with chloroform, shaking vigorously, using a 10 c.c. and a 7 c.c. portion which will bring the total volume of the extract to the mark of a 15 c.c. graduated tube. Mix thoroughly. Run at the same time and in the same way a standard using 1 c.c. of a solution of indol 0.1 mg. of indol per c.c. Compare the extract with this standard in a colorimeter, using the standard ordinarily at the 30-mm. mark. Calculate the indol to the basis of milligrams of indol per gram of moist feces.

Indol and naphthaquinone solutions should be freshly prepared or may be kept in the ice-box for some days. Indol distillates should be kept in the ice-box if not used at once, especially in hot weather. The feces must be fresh.

20. Quantitative Determination of Fat in Feces.—*Principle.*—The determination of fat in dried feces is a more or less tedious process, and one which is somewhat dangerous if applied to pathological feces. Most of the methods for the determination of fat in the moist feces are accurate, but require a long time. Saxon[1] has proposed a method for the determination of fat in moist feces, which is speedy, convenient, and accurate. The soaps of the feces are converted into free fatty acids by means of hydrochloric acid, and the material is then extracted by shaking with ether. The ether removes the neutral fat, the fatty acids which were present as such, the fatty acids derived from the soaps, and the cholesterol. The ether is removed by distillation, the crude fat purified by means of petroleum ether, and the weight of the total fat obtained. The fat is then dissolved in benzene and titrated with tenth-normal sodium alcoholate solution, using phenolphthalein as an indicator. The fatty acid is calculated, from the titration, to stearic acid.

Procedure.—Place about 5 grams (accurately weighed) of the thoroughly mixed feces in a 100 c.c. glass-stoppered graduated cylinder.[2]

[1] Saxon: *J. Biol. Chem.*, 17, 99, 1914.

[2] Care must be taken not to smear the neck of the cylinder. This may be avoided by removing the feces from the weighing bottle by means of a glass rod, the end of which is flattened, and bent in the shape of a hoe, and transferring small bits of the feces from the hoe to the cylinder, using short pieces of glass rod, which are dropped into the cylinder together with the feces.

Smith, Miller and Hawk (*Jour. Biol. Chem.*, 21, 395, 1915) have shown that the fat of feces decreases on standing even in the frozen condition. Analyses should be made on fresh feces.

Add 20 c.c. of distilled water, 1 to 2.5 c.c. of concentrated hydrochloric acid (depending upon the amount of the sample) and again, sufficient distilled water to make a total bulk of 30 c.c. Add exactly 20 c.c. of ether, stopper, and shake vigorously for five minutes. Allow to stand for a few seconds, remove the stopper, add exactly 20 c.c. of 95 per cent alcohol, and again shake for five minutes.

Stand the cylinder aside. The ether, containing practically all of the fat, will come to the top as a colored transparent layer. Blow the ether layer off into a tall 150–200 c.c. beaker.[1] The thin layer of ether which remains is diluted with 5 c.c. of ether, the tube slightly agitated, and the ether blown off. This is done in all five times, care being taken each time to wash down the sides of the cylinder. The stopper should also be washed.

Twenty c.c. of ether are again added, and the cylinder shaken for five minutes and set aside. When the ether has nearly stratified, blow it off and wash as before. During the second washing stratification will complete itself.

Evaporate the ether[2] until no trace of the alcohol, which has been carried over with it, remains. To the residue add 30 c.c. of low-boiling petroleum ether (should boil below 60°C.), and allow to stand over night. Petroleum ether for this work should be frequently tested for a residue on evaporation. If a residue is left, the ether should be redistilled.

Filter the petroleum ether solution of the fat, catch the filtrate and washings in a tall, weighed, 100 c.c. beaker, evaporate off the solvent, dry the beaker at 90°C., desiccate and weigh.

After weighing, dissolve the contents of the beaker in 50 c.c. of benzol, heat almost to the boiling-point, add 2 drops of a 0.5 per cent solution of phenolphthalein, and titrate with a decinormal solution of sodium alcoholate.[3]

Calculations.—The weight of total fat is obtained by subtracting the weight of the empty beaker from the weight of the beaker plus the dried fat. The weight of fatty acids (in terms of milligrams of stearic acid) is obtained by multiplying the number of cubic centimeters of decinormal sodium alcoholate solution by the factor 28.4. The difference between the weight of total fat and the weight of fatty acids is the weight of neutral fat in the sample extracted.

A separate determination without the addition of hydrochloric acid may be run upon the sample, for the purpose of determining the weight of neutral fat and free fatty acids. The difference between this weight and the weight of total fat is the weight of fatty acid present in the original sample in the form of soaps.

[1] This is accomplished in the same manner that water is blown from a wash bottle. The submerged end of the delivery tube is bent upward, as in the apparatus used for the determination of fat in milk by Meig's method (see Milk). This avoids upward currents which would disturb the subjacent alcohol-ether-feces layer.

[2] Erlenmeyer flasks of about 200 c.c. capacity may be used, instead of beakers, for the collection of the ether blown from the cylinders. The ether may then be distilled and recovered. The same procedure may be followed in removing the petroleum ether.

[3] In the preparation of the sodium alcoholate solution, absolute alcohol and freshly cut, bright, metallic sodium are used; otherwise the procedure is the same as that for the standardization and preparation of any alkali solution. See Reagents of Solutions, Appendix.

CHAPTER XVII

BLOOD AND LYMPH

Blood is composed of four types of form-elements (erythrocytes or red blood corpuscles, leucocytes or white blood corpuscles, blood plates or plaques and blood dust or hemoconein) held in suspension in a fluid called *blood plasma*. These form-elements compose about 60 per cent of the blood, by weight. Ordinarily blood is a dark red opaque fluid due to the presence of the red blood corpuscles, but through the action of certain substances, such as water, ether, or chloroform, it may be rendered transparent. Blood so altered was formerly said to be *laked*. The term *hemolysis* is now used in this connection and substances which cause such action are spoken of as *hemolytic agents*. The hemolytic process is simply a liberation of the hemoglobin from the stroma of the red blood corpuscle. Normal blood is alkaline in reaction to litmus, the alkalinity being due principally to sodium carbonate. When examined according to physico-chemical methods the blood is found to be very faintly alkaline (pH = 7.35). In other words it has a hydrogen ion concentration less than that of water. Even in cases of the most pronounced *acidosis* the reaction of the blood is but slightly altered (see Chapter XIX). The specific gravity of the blood of adults ordinarily varies between 1.045 and 1.075. It varies somewhat with the sex, the blood of males having a rather higher specific gravity than that of females of the same species. Under pathological conditions also the density of the blood may be very greatly altered. The freezing-point (Δ) of normal blood is about $-0.56°$C. Variations between $-0.51°$ and $-0.62°$C. may be due entirely to dietary conditions, but if any marked variation is noted it can in most cases be traced to a disordered kidney function. The total amount of blood in the body has been variously estimated at from one-twelfth to one-fourteenth of the body weight. Perhaps 1/13.5 is the most satisfactory figure. Abderhalden and Schmidt[1] have suggested a unique method for the determination of this value. It is based upon the change in the optical activity of the blood upon injection of a body of known optical activity, such, for example, as dextrin. Keith, Rowntree and Geraghty[2] have made use of a dye in the determination of blood volume.

Among the most important constituents of blood plasma are the four protein bodies,[3] *serum albumin*, *euglobulin*, *psuedoglobulin* and *fibrinogen*. *Nucleoprotein* is also present. Plasma contains about 8.2 per

[1] Abderhalden and Schmidt: *Zeit. physiol. chem.*, 66, 120, 1910.
[2] Keith, Rowntree and Geraghty: *Arch. Int. Med.*, 16, 509, 1915
[3] Howe: *Physiol. Reviews*, 5, 439, 1925.

cent of solids of which the protein constituents named above constitute approximately 84 per cent and the inorganic constituents (mainly clorides, phosphates and carbonates) approximately 10 per cent. Among the inorganic constituents sodium chloride predominates. To prevent coagulation, blood plasma is ordinarily studied in the form of an oxalated or salted plasma. (See p. 354.)

Fibrinogen is an important constituent of the plasma. It is also found in lymph and chyle as well as in certain exudates and transudates. Fibrinogen possesses the general properties of the globulins, but differs from serum globulin in being precipitated upon half-saturation with sodium chloride. In the process of coagulation of the blood the fibrinogen is transformed into fibrin. This fibrin is one of the principal constituents of the ordinary blood clot. It is claimed[1] that fibrin has the same percentage composition irrespective of the source of the blood, *e.g.*, cattle, sheep, swine, etc. The fibrinogens of the mammalian bloods that have been studied possess antigenic elements more or less common to all.[2]

The nucleoprotein of blood possesses many of the characteristics of serum globulin. In common with this body it is easily soluble in sodium chloride, and is completely precipitated from its solutions upon saturation with magnesium sulphate. It is much less soluble in dilute acetic acid than serum globulin, and its solutions coagulate at 65°–69°C.

The body formerly called serum globulin is not an individual substance. It may be resolved into two individual bodies called *euglobulin* and *pseudoglobulin*. The euglobulin is practically insoluble in water and may be precipitated in the presence of 33 per cent of saturated ammonium sulphate solution. The pseudo-globulin, on the contrary, is soluble in water and is only precipitated by ammonium sulphate in the presence of from 33 to 46 per cent of saturated ammonium sulphate solution. Investigations by Howe[3] seem to indicate the presence in serum of *two* globulins in addition to euglobulin, namely *pseudoglobulin* I and *pseudoglobulin* II whose precipitations are completed at approximately 17.4 and 21.5 per cent. sodium sulphate respectively.

Hektoen and Welker[4] have shown that euglobulin, pseudoglobulin, and serum albumin possess specific immunological properties and are hence chemically distinct. They explain the behavior of the globulins and the difficulty of separation by assuming that these two proteins form a sliding scale of chemical compounds. Sørensen has reached a similar conclusion.[5]

[1] Gortner and Wuertz: *Jour. Am. Chem. Soc.*, 39, 2239, 1917.
[2] Hektoen and Welker: *J. Am. Med. Assn.*, 85, 434, 1925.
[3] Howe: *Jour. Biol. Chem.*, 49, 93, 1921.
[4] Hektoen and Welker: Private communication.
[5] Sørensen: *J. Am. Chem. Soc.*, 47, 457, 1925.

Serum albumin differs from egg albumin in being more levorotatory, in being rendered less insoluble by alcohol, and in the fact that when precipitated by hydrochloric acid it is more easily soluble in an excess of the reagent.

When blood coagulates and the usual clot forms, a light yellow fluid exudes. This is blood *serum*. It differs from blood plasma in containing a large amount of *fibrin ferment*, a body of great importance in the coagulation of the blood, and also in possessing a lower protein content. The protein material present in plasma and not found in serum is the fibrinogen which is transformed into fibrin in the process of coagulation and removed. The specific gravity of the serum of human blood varies between 1.026 and 1.032. If blood be drawn into a vessel and allowed to remain without stirring or agitation of any sort the major portion of the red corpuscles will sink away from the upper surface, causing this portion of the clot to assume a lighter color due to the predominance of leucocytes. This light-colored portion of the clot is called the "buffy coat."

Beside the protein constituents already mentioned, other bodies which are found in both the plasma and serum are the following: *Sugar* (*glucose*), *uric acid* (*urates*), *urea*, *fat*, *amino-acids*, *enzymes*, *lecithin*, *creatine*, *carbamic acid*, *cholesterol and its esters*, *nucleo-protein*, *acetone bodies*, *paralactic acid*, *gases*, *ammonia*, *coloring-matter* (*carotin* and *xanthophylls*[1]) *and mineral constituents*. In addition to those just named the blood contains a class of substances called hormones of which *adrenalin* and *thyroxin* are the only ones whose chemical nature is definitely known. In many pathological conditions certain normal constituents are present in increased amount.

A new substance[2] has recently been isolated from pig's blood, which interferes with the determination of uric acid. Benedict has shown that it contains sulphur, and has called it *thiasine*.

The glucose content of normal human blood ranges from 70 to 120 mg. per 100 c.c. of blood[3] depending on the method used in its determination. That the diet influences the sugar content is shown by the fact that two and one-half to four hours after a meal the sugar content may increase to 180 mg. per 100 c.c. of blood.[4] In cases of hyperglycemia the blood sugar may increase to 0.3–1.0 per cent. For the quantitative determination of blood sugar see pages 381–387.

[1] Palmer, "Carotinoids and Related Pigments," New York, 1922.

[2] Hunter and Eagles: *Jour. Biol. Chem.*, 65, 623, 1925. See also Benedict. *Jour. Biol. Chem.*, 64, 215, 1925. Benedict, Newton and Behre: *Jour. Biol. Chem.*, 67, 267, 1926.

[3] Benedict: *Jour. Biol. Chem.*, 64, 207, 1925.
Folin: *Jour. Biol. Chem.*, 67, 357, 1926.

[4] Hirsch: *Zeit. physiol. Chem.*, 93, 355, 1915.

The determination of the cholesterol content of the blood is of clinical importance. Normal blood contains 150–190 mg. per 100 c.c. of blood, or about 0.16 per cent. This value may be increased (*hypercholesterolemia*) in *gall stones*, *pregnancy*, *nephritis*, *diabetes*, *arteriosclerosis*, and *syphilis*. (See page 391 for quantitative method.)

Uric acid is present in normal blood to the extent of about 2–3.5 mg. per 100 c.c. of blood. In *gout* this value may be increased to 4–10 mg. The quantitative determination of the uric acid content of blood is of importance as an aid in differentiating gout and certain other disorders exhibiting similar clinical symptoms (for methods see page 377).

The *non-protein nitrogen* of normal blood amounts to about 25–30 mg. per 100 c.c. of blood. The *urea* forms about 50 per cent of this, *uric acid* 2 per cent, *ammonia* 0.3 per cent, and *amino-acids*, and the "*undetermined nitrogen*" fraction, about 46 per cent. In *nephritis* the non-protein nitrogen of the blood is much increased. Analysis of the blood in a fatal case of *uremia* has shown a non-protein nitrogen value above 400 mg.[1]

Amino-acids are always present in the blood (5 to 8 mg. amino acid nitrogen per 100 c.c. of blood). They result for the most part from the digestion of protein material in the intestine.

The *creatine* content of normal blood averages about 5 mg. per 100 c.c. of blood. The creatine values have no important pathological significance as far as is known at the present time.

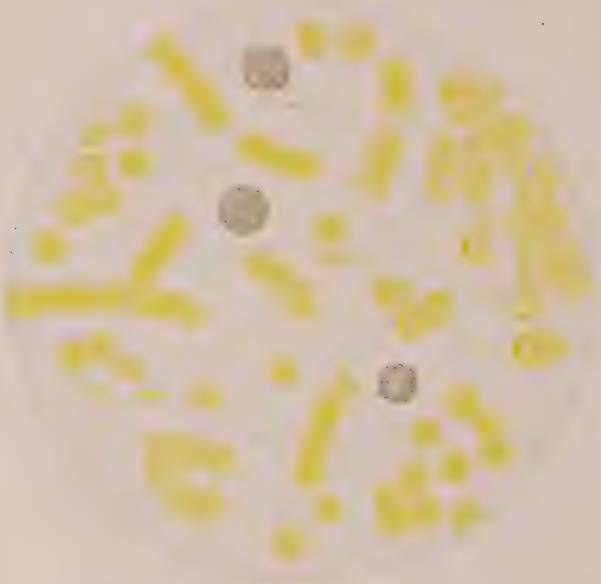
The acetone (acetone, β-hydroxybutyric acid and acetoacetic acid) content of normal blood ranges from 1.3 to 2.6 mg. per 100 c.c. of blood. In mild diabetes mellitus the value rises to 5–12 mg., whereas in severe diabetes mellitus (coma) as much as 20–45 mg. per 100 c.c. of blood serum has been found.

Normal blood contains about 20 per cent of *solids* and 3 per cent of *total nitrogen*, whereas *chlorides* are present to the extent of about 0.50 per cent. In severe *diabetes* the chlorides are *decreased* because of the accompanying diuresis. High chloride values occur in nephritis due to retention.

Abel[3] and associates have devised a method by which diffusible substances may be removed from the blood of a living animal. The process is termed *vividiffusion* and is brought about by permitting the blood from an artery to flow through collodion tubes surrounded by physiological salt solution. The dialyzable substances, except sodium

[1] Weiss and Hamilton: Reported before College of Physicians, Philadelphia, May, 1921.
[2] Folin and Denis: *Jour. Biol. Chem.*, 17, 487, 1914.
Myers and Fine: *Jour. Biol. Chem.*, 20, 391, 1915.
[3] Abel, Rowntree and Turner: *Transactions of the Ass'n of American Physicians*, 1913; also *Jour. of Pharm. and Exp. Therap.*, 5, 275, 1914.

PLATE V.



Normal Erythrocytes and Leucocytes.

chloride, are removed and the dialyzed blood is returned to the body of the animal by means of a vein. The apparatus has been modified by McGuigan and von Hess.[1]

In the application of blood-letting or venesection it has been customary to discard both the corpuscles and plasma of the withdrawn blood. Abel[2] and associates have found it possible to separate the corpuscles from the removed blood by centrifugation and to return them to the body suspended in Locke's solution. They name the procedure *plasmaphæresis*. By this means blood-letting can be carried out repeatedly during a short interval of time without endangering the life of the animal.

There has been considerable controversy regarding the form of the erythrocytes or red blood corpuscles of human blood. It is claimed by some investigators that the cells are *bell-shaped* or *cup-shaped*. As the erythrocytes occur normally *in the circulation*, however, they are probably thin, non-nucleated, biconcave discs.[3]

The blood of most mammals contains erythrocytes similar in form to those of human blood. In the blood of birds, fishes, amphibians and reptiles the erythrocytes are ordinarily more or less elliptical, biconvex and possess a nucleus. The erythrocytes vary in size with the different animals. The average diameter of the erythrocytes of blood from various species is given in the following table:[4]

Elephant	$\frac{1}{2738}$ of an inch.
Guinea-pig	$\frac{1}{3283}$ of an inch.
Man	$\frac{1}{3250}$ of an inch.
Monkey	$\frac{1}{3382}$ of an inch.
Dog	$\frac{1}{3581}$ of an inch.
Rat	$\frac{1}{3682}$ of an inch.
Rabbit	$\frac{1}{3653}$ of an inch.
Mouse	$\frac{1}{3748}$ of an inch.
Lion	$\frac{1}{4148}$ of an inch.
Ox	$\frac{1}{4219}$ of an inch.
Horse	$\frac{1}{4248}$ of an inch.
Pig	$\frac{1}{4288}$ of an inch.
Cat	$\frac{1}{4872}$ of an inch.
Sheep	$\frac{1}{4912}$ of an inch.
Goat	$\frac{1}{6189}$ of an inch.
Musk-deer	$\frac{1}{12825}$ of an inch.

The erythrocytes, from whatever source obtained, consist essentially of two parts, the *stroma* or protoplasmic tissue and its enclosed pigment, *hemoglobin*. For human blood the number of erythrocytes present in the fluid as obtained from well-developed males in good physical condi-

[1] McGuigan and Von Hess: *Jour. Pharm. and Exp. Therap.*, vol. 6, 1914.

[2] Abel, Rowntree, Turner, Marshall and Lamson (see Abel's Mellon Lecture," 1915): also Abel: *Science*, 42, 135, 1915.

[3] When examined singly under the microscope, they possess a pale greenish-yellow color (see Plate IV, opposite p. 336), whereas when grouped in large masses a reddish tint is noted.

[4] Wormley's Micro-Chemistry of Poisons, second edition, p. 733.

tion is about 5,500,000 per cubic millimeter.[1] The normal content of the blood of adult females is from 4,000,000 to 4,500,000 per cubic millimeter. The number of erythrocytes varies greatly under different conditions. For instance, the number may be increased after the transfusion of blood of the same species of animals; by residing in a high altitude; or as a result of strenuous physical exercise continued over a short period of time. An increase is also noted in starvation; after partaking of food; after cold or hot baths; after massage, in partial asphyxia, and after fright, as well as after the administration of certain drugs and accompanying certain diseases, such as cholera, diarrhea, dysentery and yellow atrophy of the liver. A decrease in the number occurs in the different forms of anemia. The number has been known to increase to 7,040,000 per cubic millimeter as a result of physical exercise, while 11,000,000 per cubic millimeter have been noted in cases of polycythemia and increases nearly as great in cyanosis. The number has been known to decrease to 500,000 per cubic millimeter or lower in pernicious anemia.

Erythrocytes possess the property, when properly treated, of "clumping" together in masses and precipitating, producing so-called *agglutination*. Cells other than erythrocytes (*e.g.*, bacteria) possess this property. When spoken of in connection with the blood such action is termed *hemagglutination*. A substance which will bring about hemagglutination is said to contain *hemagglutinins*. These hemagglutinins are particularly abundant in the vegetable kingdom.[2] For a demonstration of hemagglutination see page 347.

Oxyhemoglobin, the coloring matter of the blood, is a conjugated protein. Through treatment with hydrochloric acid it may be split into a protein body called *globin*, and *hemochromogen*, an iron-containing pigment. The latter body is rapidly transformed into *hematin* in the presence of oxygen, and this in turn gives place to hematin-hydrochloride or *hemin* (Figs. 103 and 104, page 350). The pigment of arterial blood is for the most part loosely combined with oxygen and is termed *oxy*-hemoglobin, whereas the pigment of venous blood is principally hemoglobin (so-called *reduced* hemoglobin). Oxyhemoglobin is the oxygen carrier of the body and belongs to the class of bodies known as respiratory pigments. It is held within the stroma of the erythrocyte. The reduction of oxyhemoglobin to form hemoglobin (so-called *reduced* hemoglobin) occurs in the capillaries. Oxyhemoglobin may be crystallized

[1] This statement is based upon observations made upon the blood of athletes in training. See Hawk: *Amer. Jour. Physiol.*, 10, 384, 1904. It is generally stated in text-books that the blood of males contains about 5,000,000 per cubic millimeter.

[2] Mendel: *Archivio di fisiologia*, 7, 168, 1909; also Schneider: *Journal of Biological Chem.*, 11, 47, 1912.

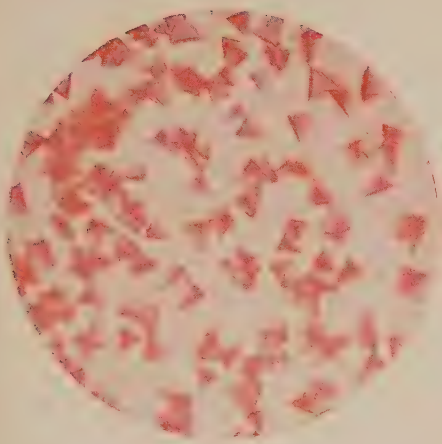


FIG. 95.—OXYHEMOGLOBIN CRYSTALS FROM BLOOD OF THE GUINEA-PIG.[1]

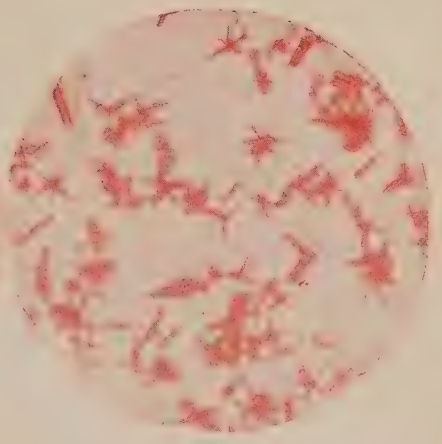


FIG. 96.—OXYHEMOGLOBIN CRYSTALS FROM BLOOD OF THE RAT.[1]

FIG. 97.—OXYHEMOGLOBIN CRYSTALS FROM BLOOD OF THE HORSE.[1]

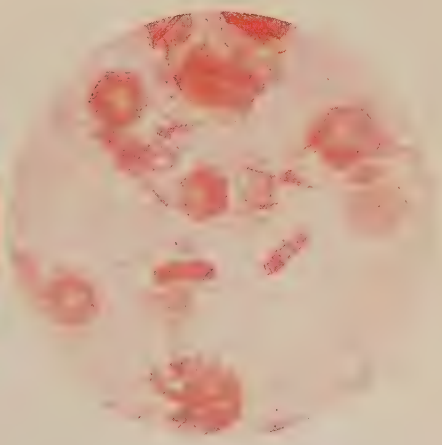


FIG. 98.—OXYHEMOGLOBIN CRYSTALS FROM BLOOD OF THE SQUIRREL.[1]

[1] The crystals in Figs. 95–101 were reproduced from micro-photographs furnished by Prof. E. T. Reichert, of the University of Pennsylvania.

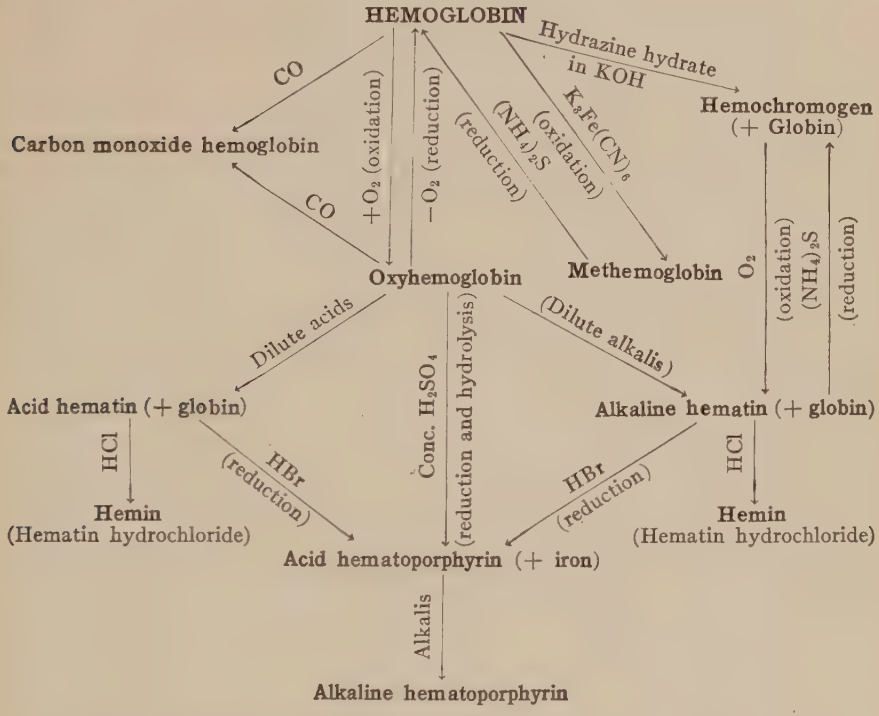
FIG. 99.—OXYHEMOGLOBIN CRYSTALS FROM BLOOD OF THE DOG.[1]

FIG. 100.—OXYHEMOGLOBIN CRYSTALS FROM BLOOD OF THE CAT.[1]

FIG. 101.—OXYHEMOGLOBIN CRYSTALS FROM BLOOD OF THE NECTURUS.[1]

[1] The crystals in Figs. 95–101 were reproduced from micro-photographs furnished by Prof. E. T. Reichert, of the University of Pennsylvania.

and a specific form of crystal obtained from the blood of each individual species (see Figs. 95 to 101, pages 337 and 338). This fact seems to indicate that there are many varieties of oxyhemoglobin. The interesting findings of Reichert and Brown are of great value in this connection. These investigators prepared oxyhemoglobin crystals from the blood of over *one hundred* species of animals and subsequently studied the characteristics of the crystals very minutely from the standpoint of crystallography. Their findings may prove of importance from the standpoint of heredity and the origin of species.



The following bodies may be derived from hemoglobin, and each possesses a specific absorption spectrum which serves as an aid in its detection and identification: Oxyhemoglobin, methemoglobin, carbon-monoxide hemoglobin, nitric-oxide hemoglobin, hemochromogen, hematin, acid-hematin, alkali-hematin and hematoporphyrin (see Absorption Spectra, Plates I and II).

The relationship between hemoglobin and its derivatives may be represented by the scheme shown above.

According to a recent determination[1] the molecular weight of hemoglobin in aqueous solution is 66,800. This total weight is believed

[1] Svedberg and Fahraeus: *Jour. Am. Chem. Soc.*, 48, 430, 1926.

to be made up of four groups each possessing a molecular weight of 16,700.

The chemical transformations which occur in the blood during *respiration* are complicated and of great importance. In brief the exchange of oxygen and carbon dioxide may be described as follows: Oxygen from the air passes through the lungs into the blood where it is appropriated for the most part by the red blood corpuscles. The hemoglobin of these corpuscles possesses the property of uniting with oxygen, forming *oxyhemoglobin*. This oxyhemoglobin possesses a red color and imparts to the *arterial blood* its bright appearance. The oxygen is thus borne by these blood cells in the circulating blood to all parts of the body. As the blood passes through the capillaries it gives up part of its oxygen which is used by the tissues in their varied activities. As the blood loses its oxygen it becomes darker in color due to the fact that the oxyhemoglobin has been transformed into hemoglobin (or reduced hemoglobin). At the same time in the tissue capillaries the blood takes up excretory products from the tissues, the chief of which is *carbon dioxide*. This carbon dioxide is present in the blood mainly as carbonic acid and sodium acid carbonate; a small amount is probably combined with the proteins of the plasma. We now have so-called venous blood. This is, in turn, carried to the lungs where the carbon dioxide is exchanged for oxygen and the cycle is repeated. (For further discussion of *respiratory exchange*, see Chapter XIX.)

The white corpuscles (or leucocytes) of human blood differ from the red corpuscles (or erythrocytes) in many particulars, such as being somewhat larger in size, in containing at least a single nucleus and in possessing ameboid movement (see Plate IV, opposite page 336). They are typical animal cells and therefore contain the following bodies which are customarily present in such cells: *Proteins*, *fats*, *glycogen*, *purine bodies*, *enzymes*, *phosphatides*, *lecithin*, *cholesterol*, *inorganic salts*, and *water*. Compound proteins make up the chief part of the protein quota of leucocytes, the nucleoproteins predominating. Of the enzymes present the proteolytic are the most important. It is claimed that there are two proteolytic enzymes in leucocytes, one active in alkaline solution and present in the polynuclear cells,[1] and the other active in acid medium and present in mononuclear cells. It is claimed that the granular leucocytes originate in the bone marrow, whereas the non-granular leucocytes (lymphocytes) have a lymphatic origin (lymph glands or lymphoid tissue); this matter of origin is uncertain.

[1] For discussion of different types of leucocytes see Webster's "Diagnostic Methods" or some similar volume.

The normal number of leucocytes in human blood varies between 5000 and 10,000 per cubic millimeter. The ratio between the leucocytes and erythrocytes is about 1 : 350–500. A *leucocytosis* is said to exist when the number of leucocytes is increased for any reason. Leucocytoses may be divided into two general classes, the *physiological* and the *pathological*. Under the physiological form would be classed those leucocytoses accompanying pregnancy, parturition and digestion, as well as those due to mechanical and thermal influences. The leucocytoses spoken of as pathological are the inflammatory, infectious, post-hemorrhagic, toxic and experimental forms, as well as the type of leucocytosis which accompanies malignant disease.

The blood plates (platelets or plaques) are round or oval colorless discs which possess a diameter about one-third as great as that of the erythrocytes. Upon treatment with certain reagents, *e.g.*, artificial gastric juice, they may be separated into a homogeneous, non-refractive portion and a granular, refractive portion. The blood plates are associated with the coagulation of the blood. This relationship is not completely understood at present.

The hemoconein or so-called "blood dust" is made up of round granules which usually have a diameter somewhat less than 1 micron. The serum of normal as well as of pathological blood contains these granules. They were first described by Müller to whom they appeared as highly refractile granules possessed of Brownian movement. The "blood dust" is apparently not concerned with the coagulation of the blood. The granules are insoluble in alcohol, ether and acetic acid and are not blackened by osmic acid. According to Müller, the granules making up the so-called "blood dust" constitute a new organized constituent of the blood, whereas other investigators believe them to be merely free granules from certain of the forms of leucocytes. They appear to possess no clinical significance.

The processes involved in the coagulation of the blood are not fully understood.[1] Several theories have been advanced and each has its adherents. The literature on the subject is confused by the introduction of new terms for the same substances. The theory which appears to be fully as firmly founded upon experimental evidence as any is substantially the following: Blood contains a zymogen called *prothrombin* which combines with the calcium salts present to form an enzyme known as *thrombin* or *fibrin-ferment*. When blood is drawn from a vessel the fibrin-ferment at once acts upon the fibrinogen present and gives rise to the formation of *fibrin*. This fibrin forms in shreds

[1] For a discussion of the various theories concerning blood clotting, see Mathews, "Physiological Chemistry," 4th ed., 1925, pp. 533 and 1066.

throughout the blood mass and, holding the form elements of the blood within its meshes, serves to produce the typical *blood clot.* The fibrin shreds gradually contract, the clot assumes a jelly-like appearance and the yellowish serum exudes. If, immediately upon the withdrawal of blood from the body, the fluid be rapidly stirred or thoroughly "whipped" with a bundle of coarse strings, twigs, or a specially constructed beater, the fibrin shreds will not form in a network throughout the blood mass but instead will cling to the device used in beating. In this way the fibrin may be removed and the remaining fluid is termed *defibrinated* blood. The above theory of the coagulation of the blood may be stated briefly as follows:

I. Prothrombin + Calcium Salts = Thrombin (or Fibrin-ferment).

II. Thrombin (or Fibrin-ferment) + Fibrinogen = Fibrin.

Howell[1] has suggested an ingenious modification of the above theory. He says: "In the circulating blood we find as constant constituents *fibrinogen, prothrombin, calcium salts* and *antithrombin.* The last-named substance holds the prothrombin in combination and thus prevents its conversion or activation to thrombin. When the blood is shed, the disintegration of the corpuscles (platelets) furnishes material (thromboplastin) which combines with the antithrombin and liberates the prothrombin; the latter is then activated by the calcium and acts on the fibrinogen. According to this view the actual process of coagulation involves only three factors, fibrinogen, prothrombin and calcium. These three factors exist normally in the circulating blood but are prevented from reacting by the presence of antithrombin."

The question as to whether *menstrual blood* coagulates has caused much discussion. Some experiments seem to show that it *does not coagulate* because of the *removal of fibrin-ferment and fibrinogen* from such blood by the endometrium or lining membrane of the uterus.[2]

Among the medico-legal tests for blood are the following: (1) Microscopical identification of the erythrocytes, (2) spectroscopic identification of blood solutions, (3) the guaiac test, (4) the benzidine reaction, (5) preparation of hemin crystals, (6) Bordet reaction ("biological" blood test). This last test is the most satisfactory medico-legal test for blood.

Up to within recent times it has been impossible to make an absolute differentiation of human blood. The so-called "biological" blood test has, however, made such a differentiation possible. This test, known as the Bordet reaction, is founded upon the fact that the blood serum of an

[1] Howell: *American Journal of Physiology,* 29, 187, 1911. Text Book of Physiology, 1925.
[2] Bell: *Jour. Path. and Bact.,* 18, No. 4, 1914

animal into which has been injected the blood of another animal of different species develops the property of agglutinating and dissolving erythrocytes *similar to those injected*, but exerts this influence upon the blood from *no other species*. The antiserum used in this test is prepared by injecting rabbits with 5–10 c.c. of human defibrinated blood, at intervals of about four days, until a total of between 50 and 80 c.c. has been injected. After a lapse of one or two weeks the animal is bled, the serum collected, placed in sterile tubes and preserved for use as needed. In examining any specific solution for human blood it is simply necessary to combine the antiserum and the solution under examination in the proportion of 1:100 and place the mixture at 37°C. If human blood is present in the solution a turbidity will be noted and this will change within three hours to a distinctly flocculent precipitate. This antiserum will react thus with no other known substance.

Of the other five blood tests mentioned the last two named are generally considered to be the most satisfactory. They give equally reliable results with fresh blood and with blood from clots or stains of long standing, provided the latter have not been exposed to a high temperature or to the rays of the sun for a long period. The technic of the tests is simple and the formation of the dark brown or chocolate-colored crystals of hemin or the production of the green or blue color with benzidine is indisputable proof of the presence of blood in the fluid, clot or stain examined. The weak point of the tests, medico-legally, lies in the fact that they do not differentiate between human blood and that of certain other species of animals.

The guaiac test (see page 347), although generally considered less accurate than the hemin test, is held by some to be a more delicate test than the hemin test, if properly performed. One of the most common mistakes in the manipulation of this test is the use of a guaiac solution which is too concentrated and which, when brought into contact with the aqueous blood solution, causes the separation of a voluminous precipitate of a resinous material which may obscure the blue coloration; this is particularly true of the test when used for the examination of blood stains. A solution of guaiac made by dissolving 1 gram of the resin in 60 c.c. of 95 per cent alcohol is very satisfactory for general use. The test is frequently objected to upon the ground that various other substances, *e.g.*, milk, pus, saliva, etc., respond to the test and that it cannot therefore be considered a specific test for blood and is of value only in a negative sense. We have demonstrated to our own satisfaction, however, that many samples of milk give the blue color upon the addition of an alcoholic solution of guaiac resin without the addition of

hydrogen peroxide or old turpentine. It has also been shown[1] that those milks which respond positively, fail to do so after boiling. In the case of blood the test is positive both before and after boiling the blood for 15–20 seconds. Pus does not respond after boiling. Old, partly putrified pus gives the test even without the addition of hydrogen peroxide or old turpentine, whereas fresh pus responds upon the addition of hydrogen peroxide. Saliva gives a positive reaction only in case blood or pus is present. Certain plant extracts give the test before but not after boiling for 15–20 seconds. Buckmaster has advocated the use of an alcoholic solution of guaiaconic acid instead of an alcoholic solution of guaiac resin. He claims that he was able to produce the blue color upon the addition of the guaiaconic acid to milk *only* when the sample of milk tested was brought from the country in *sterile bottles*, and further, that no sample of London milk which he examined responded to the test. In the application of the guaiac test to the detection of blood, he states that he was able to detect *laked blood* when present in the ratio 1:5,000,000 and *unlaked blood* when present in the ratio 1:1,000,000. This author considers the guaiac test to be far more trustworthy than is generally believed.

Lymph may be considered as the "middle man" in the transactions between blood and tissues. It is the medium by which the nutritive material and oxygen transported by the blood for the tissues is brought into intimate contact with those tissues and thus utilized. In the further fulfillment of its function, the lymph bears from the tissues water, salts and the products of the activity and catabolism of the tissues and passes these into the blood. Lymph, therefore, exercises the function of a "go-between" for blood and tissues. It bathes every active tissue of the animal body, and is believed to have its origin partly in the blood and partly in the tissues.

In chemical characteristics, lymph resembles blood plasma. In fact, it has been termed "blood without its red corpuscles." Lymph from the thoracic duct of a fasting animal or from a large lymphatic vessel of a well-nourished animal is of a variable color (colorless, yellowish or slightly reddish) and alkaline in reaction to litmus. It contains fibrinogen, fibrin-ferment and leucocytes and coagulates slowly, the clot being less firm and bulky than the blood clot. Serum albumin and serum globulin are both present in lymph, the albumin predominating in a ratio of about 3 or 4:1. The principal inorganic salts are sodium salts (chloride and bicarbonate), whereas the phosphates of potassium, calcium, magnesium and iron are present in smaller amount.

Substances which stimulate the flow of lymph are termed *lympha-*

[1] Leary: Private communication.

gogues. Such substances as sugar, urea, certain salts (especially sodium chloride), peptone, egg albumin, extracts of dogs' liver and intestine, crab muscles and blood leeches are included in this class.

In a fasting animal, the lymph coming from the intestine is a clear, transparent fluid possessing the characteristics already outlined. After a meal containing fat has been ingested, this intestinal lymph is white or "milky." This is termed *chyle* and is essentially lymph possessing an abnormally high (5–15 per cent) content of emulsified fat. This chyle is absorbed by the lacteals of the intestine and transported to the lower portion of the thoracic duct. Apart from the fat content, the composition of lymph and chyle are similar.

Experiments on Blood

I. Defibrinated Ox-blood

1. Reaction.—Moisten red and blue litmus papers with 10 per cent sodium chloride solution and test the reaction of the defibrinated blood. Test by Congo red paper also.

2. Microscopical Examination.—Examine a drop of defibrinated blood under the microscope. Compare the objects you observe with Plate IV, opposite page 336. Repeat the test with a drop of your own blood.

3. Specific Gravity.—Determine the specific gravity of defibrinated blood by means of an ordinary specific gravity spindle. Compare this result with the specific gravity as determined by Hammerschlag's method in the next experiment.

4. Specific Gravity by Hammerschlag's Method.—Fill an ordinary urinometer cylinder about one-half full of a mixture of chloroform and benzene, having a specific gravity of approximately 1.050. Into this mixture allow a drop of the blood under examination to fall from a pipette or directly from the finger in case fresh blood is being examined. Care must be taken not to use too large a drop of blood and to keep the drop from coming into contact with the walls of the cylinder. If the blood drop sinks to the bottom of the vessel, thus showing it to be of higher specific gravity than the surrounding fluid, add chloroform until the blood drop remains suspended in the mixture. Stir carefully with a glass rod after adding the chloroform. If the blood drop rises to the surface upon being introduced into the mixture, thus showing it to be of lower specific gravity than the surrounding fluid, add benzene until the blood drop remains suspended in the mixture. Stir with a glass rod after the benzene is added. After the blood drop has been brought to a suspended position in the mixture by means of one or more additions of chloroform and benzene this final mixture should be filtered through muslin and its specific gravity accurately determined. What is the specific gravity of the blood under examination?

5. Tests for Various Constituents.—Place 10 c.c. of defibrinated blood in an evaporating dish, dilute with 50 c.c. of water and heat to boiling. Is there any coagulation, and if so what bodies form the coagulum? At the boiling-point add about 50 c.c. of very dilute acetic acid (made by adding 2 drops of 36 per cent acetic acid to 50 c.c. of water) and again heat to boiling for a few moments.

Filter. The filtrate should be clear and the coagulum dark brown. Reserve this coagulum. What body gives the coagulum this color? Evaporate the filtrate to about 25 c.c., filtering off any precipitate which may form in the process. Make the following tests upon the filtrate:

(a) Benedict's Test.—To 5 c.c. of the neutralized filtrate add 5 drops of Benedict's solution and boil one minute.

(b) Chlorides.—To a small amount of the filtrate in a test-tube add a few drops of nitric acid and a little silver nitrate. In the presence of chloride, a white precipitate of silver chloride will form.

(c) Phosphates.—Test for phosphates by nitric acid and molybdate solution according to directions given on page 109.

(d) Crystallization of Sodium Chloride.—Place the remainder of the filtrate in a watch glass and evaporate it on a water-bath. Examine the crystals under the microscope and compare them with those in Fig. 86, page 309.

6. Test for Iron.—Incinerate a small portion of the coagulum from the last experiment (5) in a porcelain crucible. Cool, dissolve the residue in dilute hydrochloric acid and test for iron by potassium ferrocyanide or ammonium thiocyanate. Which of the constituents of the blood contains the iron?

FIG. 102.—EFFECT OF WATER ON ERYTHROCYTES.

7. Hemolysis ("Laking Blood").—Note the opacity of ordinary defibrinated blood. Place a few cubic centimeters of this blood in a test-tube and add water, a little at a time, until the blood is rendered transparent. Hemolysis has taken place. How does the water act in causing this transparency? Examine a drop of hemolyzed blood under the microscope. How does its microscopical appearance differ from that of unaltered blood? What other agents may be used to bring about hemolysis?

8. Osmotic Pressure.—Place a few cubic centimeters of blood in each of three test-tubes. Hemolyze the blood in the first tube according to directions given in the last experiment (7): add an equal volume of isotonic (0.9 per cent) sodium chloride to the blood in the second tube, and an equal volume of 10 per cent sodium chloride to the blood in the third tube. Mix thoroughly by shaking and after a few moments examine a drop from each of the three tubes under the

microscope (see Figs. 102 and 219). What do you find and what is your explanation from the standpoint of osmotic pressure?

9. **Hemagglutination.**—The common garden bean, such as the Scarlet Runner,[1] contains a protein substance which exhibits the interesting property of causing a clumping or agglutination of red blood corpuscles.[2]

Dilute defibrinated blood[3] ten times with physiological sodium chloride solution (0.9 per cent) and place 1 c.c. in each of three small test-tubes.

Grind three beans in a coffee mill, or with mortar and pestle to a fine meal and extract for a few minutes with 0.9 per cent sodium chloride solution. Filter and add 0.05 c.c. (about 2–3 drops) of the filtered extract to the first of the blood tubes; 0.01 c.c. to the second; and 0.05 c.c. of 0.9 per cent sodium chloride to the third.

Invert each tube to mix the contents thoroughly, and note the rapid agglutination and precipitation of the blood corpuscles in the first tube, a less rapid agglutination in the second, while the third or control tube remains unaltered. In one-half hour the corpuscles in the first tube often are packed solid and one is able to pour off perfectly clear serum.

If the remainder of the bean extract is boiled for a few minutes, the coagulum filtered out and 0.05 c.c. of the filtrate added to the control tube, still no agglutination occurs, indicating that the hemagglutinin has been destroyed or removed by the boiling.

10. Diffusion of Hemoglobin.—Prepare some hemolyzed ("laked") blood, thus liberating the hemoglobin from the erythrocytes. Test the diffusion of the hemoglobin by preparing a dialyzer like one of the models shown in Fig. 16, page 74. How does hemoglobin differ from other well-known crystallizable bodies?

11. Guaiac Test.—By means of a pipette drop an alcoholic solution of guaiac (strength about 1:60)[4] or the Lyle-Curtman guaiac reagent[5] (see p. 321) into the solution under examination[6] until a turbidity is observed and add old turpentine or hydrogen peroxide, drop by drop, until a blue color is obtained.

In the detection of *small* amounts of blood the quantity of guaiac used should also be decreased. Do any other substances respond in a similar manner to this test? Is a positive guaiac test a sure indication of the presence of blood? (See discussion on page 343.)

[1] The Scarlet Runner is a familiar variety purchasable in every seed store. It occurs in two varieties, the *white* and the *red*. Ricin, a protein constituent of the castor bean, also possesses pronounced agglutinating properties. Because of its poisonous nature it is, however, not suitable for use in class experiments.

[2] Mendel: *Archivio di fisiologia*, 7, 168, 1909; Schneider: *Journal Biol. Chem.*, 11, 47, 1912.

[3] Rabbit's blood is especially desirable (Mendel: *loc. cit.*) and may be obtained for the purpose by bleeding from a small cut on the animal's ear and defibrinating.

[4] Buckmaster advises the use of an alcoholic solution of guaiaconic acid instead of an alcoholic solution of guaiac resin. See page 344.

[5] Lyle and Curtman; *Jour. Biol. Chem.*, 33, 1, 1918.

[6] Alkaline solutions should be made slightly acid with acetic acid, as the blue end-reaction is very sensitive to alkali.

12. Ortho-tolidin Test (Ruttan and Hardisty).[1]—**To 1 c.c. of a 4 per cent glacial acetic acid solution of o-tolidin**[2] **in a test-tube add 1 c.c. of the solution under examination and 1 c.c. of 3 per cent hydrogen peroxide. In the presence of blood a bluish color develops (sometimes rather slowly) and persists for some time (several hours in some instances).**

This test is said to be as sensitive for the detection of occult blood in feces and stomach contents as is the benzidine reaction. It is also claimed to be more satisfactory for urine than any other blood test. The acetic acid solution may be kept for one month with no reduction in delicacy.

13. Benzidine Reaction.—This is one of the most delicate of the reactions for the detection of blood. Different benzidine preparations vary greatly in their sensitiveness, however. Inasmuch as benzidine solutions change readily upon contact with light it is essential that they be kept in a dark place.

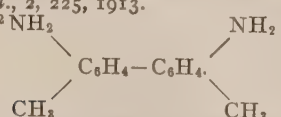
The test is performed as follows: To 3 c.c. of a saturated solution of benzidine[3] **in glacial acetic acid**[4] **add 2 c.c. of the solution to be tested and one c.c. of 3 per cent hydrogen peroxide. A positive test is indicated by a blue or green color.**

Confirmatory test. Make 10 c.c. of the solution acid with acetic acid and extract by shaking with 5 c.c. of ether. Pour off the ether into a small evaporating dish. Put on a hot water bath (with the flame turned out). Evaporate to dryness. To the residue add a few drops of water, a drop of saturated solution of benzidine in glacial acetic acid and a drop of 3 per cent hydrogen peroxide. A blue or green color indicates blood.

The hemoglobin decomposes the hydrogen peroxide (catalysis) and the liberated oxygen oxidizes the benzidine. The sensitiveness of the benzidine reaction is greater when applied to aqueous solutions than when applied to the urine. Walter[5] has also shown the test to be very delicate, and claims it to be more satisfactory than the guaiac test.

Lyle, Curtman and Marshall[6] have investigated the benzidine reaction very carefully. They suggest a new procedure in preparing the reagent and in conducting the test.

[1] Ruttan and Hardisty: *Canadian Medical Ass'n. Journal*, Nov., 1912; also *Biochemical Bull.*, 2, 225, 1913.

[2] 

[3] Merck's "Blue Label" benzidine for blood test is satisfactory. Many products are not.

[4] Glacial acetic acid is preferable, but if it is not available, alcohol acidified with acetic acid may be used.

[5] Walter: *Deut. med. Woch.*, 36, p. 309.

[6] Lyle, Curtman and Marshall: *Jour. Biol. Chem.*, 19, 445, 1914.

The test follows: Into a perfectly clean dry test-tube introduce 1.4 c.c. benzidine solution,[1] add 0.2 c.c. of water or glacial acetic acid, then 1 c.c. of the fluid to be tested and finally 0.4 c.c. of 3 per cent hydrogen peroxide. Note the appearance of a blue color, which reaches its maximum in five to six minutes.

The acetic acid keeps the benzidine in solution. An excess diminishes the delicacy of the reagent.

Hydrogen peroxide supplies oxygen for the reaction and also bleaches the blue color. An excess of peroxide interferes with the reaction by destroying the catalytic power of the blood and by reacting with the benzidine itself, with the formation of products which appear to have an inhibitory action. It is very essential that the peroxide be added *last.*

The benzidine solution should be *dilute.* Such solutions are exceedingly sensitive and permit the detection of blood when present in ratio 1:5,000,000.

Gregersen and Boas[2] claim that the use of a too concentrated benzidine solution may lead to wrong diagnosis because of the excessive sensitiveness of the reagent. The feces of normal persons on a meat-free diet often yield a positive reaction. They suggest the use of a 0.5 per cent benzidine solution and the replacement of the hydrogen peroxide by barium peroxide which is much more stable. They, however, admit that slight hemorrhages may go undiscovered when this dilute benzidine solution is used.

14. Hemin Test.—(a) Teichmann's Method.—Place a very small drop of blood on a microscopic slide, add a small drop of water and stir to lake the blood. Add a fraction of a drop of dilute (0.9 per cent) sodium chloride solution[3] and carefully evaporate to dryness over a low flame. Put a cover glass in place, run underneath it a drop of glacial acetic acid and warm gently until the formation of gas bubbles is noted. Add another drop of glacial acetic acid, cool the preparation, examine under the microscope and compare the crystals with those shown in Figs. 103 and 104.

The hemin crystals result from the decomposition of the hemoglobin of the blood. What are the steps involved in this process? The hemin crystals are also called Teichmann's crystals. Is this an *absolute* test for blood? Is it possible to differentiate between human blood and the blood of other species by means of the hemin test?

(b) Nippe's Method.[4]—Spread a small drop of blood on a slide in the form of a film and evaporate to dryness over a low flame. Now add 2 drops of a solution

[1] Benzidine solution may be prepared as follows: Place 4.33 c.c. of glacial acetic acid in a small Erlenmeyer flask, warm to 50° and add 0.5 gram of benzidine. Heat the flask for eight to ten minutes in water at 50°. To the resultant solution add 19 c.c. of distilled water. This solution may be kept for several days without deterioration.

[2] Boas: *Berl. klin. Woch.*, 56, 939, 1919.

[3] Buckmaster considers the use of potassium chloride preferable.

[4] Nippe: *Deut. med. Woch.*, 38, 2222, 1912.

FIG. 103.—HEMIN CRYSTALS FROM HUMAN BLOOD.
Reproduced from a micro-photograph furnished by Prof. E. T. Reichert, of the University of Pennsylvania.

FIG. 104.—HEMIN CRYSTALS FROM SHEEP BLOOD.
Reproduced from a micro-photograph furnished by Prof. E. T. Reichert, of the University of Pennsylvania.

containing 0.1 gram each of potassium chloride, iodide and bromide in 100 c.c. of glacial acetic acid. Place a cover-glass in position and heat gently over a low flame until gas bubbles form and the solution boils. Run 1–2 drops of the reagent underneath the cover-glass and examine under a microscope. Compare the crystals with those shown in Figs. 103 and 104.

This method is more rapid than Teichmann's method and crystals of inorganic chlorides are not formed. In Teichmann's method crystals of sodium chloride, Fig. 105, often obscure the hemin crystals.

15. Catalytic Action.—To about 10 drops of blood in a test-tube add twice the volume of hydrogen peroxide, without shaking. The mixture foams. What is the cause of this phenomenon?

16. Crystallization of Oxyhemoglobin. Reichert's Method.—Add to 5 c.c. of the blood of the dog, horse, guinea-pig, or rat, before or after laking, or defibrinating, from 1 to 5 per cent of ammonium oxalate in substance. Place a drop of this oxalated blood on a slide and examine under the microscope. The crystals of oxyhemoglobin will be seen to form at once near the margin of the drop, and in a few minutes the entire drop may be a solid mass of crystals. Compare the crystals with those shown in Figs. 95–101, pages 337 and 338.

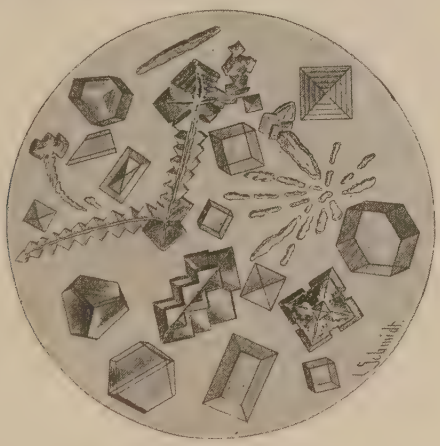


FIG. 105.—SODIUM CHLORIDE.

17. Preparation of Hemoglobin: Method of Marshall and Welker.[1] Draw blood into a flask and defibrinate by shaking with glass beads. Strain through cheese cloth. Centrifuge. Wash corpuscles 3 or 4 times with 0.9 per cent NaCl. Add ether a few drops at a time with thorough mixing until a clear solution is obtained. If the solution is viscid add a little water and then an equal volume of aluminium hydroxide cream (see "Reagents and Solutions"). Mix thoroughly and filter. Cool to 0°C. and add absolute alcohol (also cooled to 0°) to make the alcohol percentage 20–30 per cent. Let stand at a few degrees below 0°C. Wash crystals by decantation with 25 per cent alcohol at 0°C. Dry in a desiccator over sulphuric acid.

[1] Marshall and Welker: *J. Am. Chem. Soc.*, 35, 820, 1913; Welker and Williamson: *J. Biol. Chem.*, 41, 75, 1920.

18. Preparation of Hematin.—Place 100 c.c. of *hemolyzed* (*laked*) blood in a beaker and add 95 per cent alcohol until precipitation ceases. What bodies are precipitated? Transfer the precipitate to a flask and boil with 95 per cent alcohol previously acidulated with sulphuric acid. Through the action of the acid the hemoglobin is split into hematin and a protein body called globin. Later the "sulphuric acid ester of hematin" is formed, which is soluble in the alcohol. Continue heating until the precipitate is no longer colored, then filter. Partly saturate the filtrate with sodium chloride and warm. In this process the "hydrochloric acid ester of hematin" is formed. Filter and dissolve on the filter paper by sodium carbonate. Save this alkaline solution of hematin and make a spectroscopic examination later after becoming familiar with the use of the spectroscope. How does the spectrum of oxyhemoglobin differ from that of the derived *alkali hematin?*

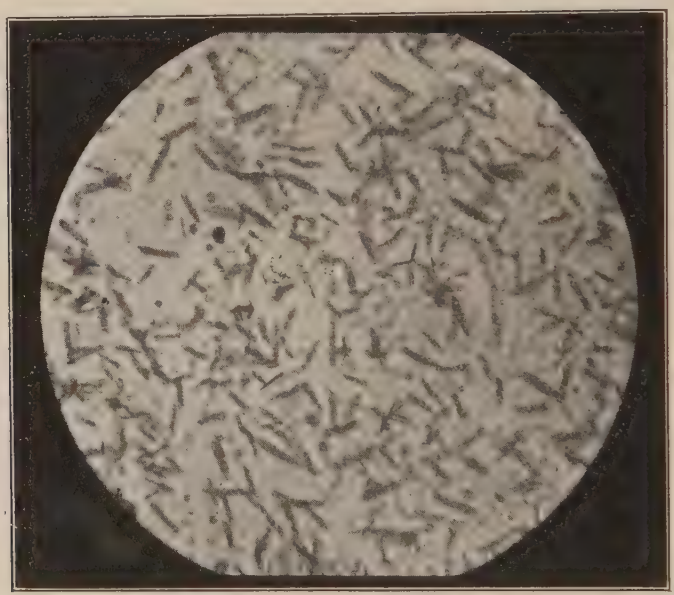


FIG. 106.—HEMOCHROMOGEN CRYSTALS.
(*Prepared and photographed by Arthur G. Cole, University of Illinois, College of Medicine, Chicago.*)

19. Hemochromogen Test.—Add 2 or 3 drops of Takayama's solution[1] to a small piece of the suspected material on a slide. Cover with a cover glass. Examine under the microscope. Salmon pink crystals should appear in 1 to 6 minutes. At the same time the color changes through green-brown, dark red to pink, indicating the formation of hemochromogen and confirming the test. The crystals have a shallow rhomboid form.

This method is simpler to use than the hemin test and may be used to confirm the latter in doubtful cases. It is not always given by old blood stains (over six months old) and hence does not replace the hemin test.[2] The glucose may act as

[1] A mixture of 10 per cent NaOH 3 c.c., pyridine 3 c.c., saturated solution of glucose 3 c.c. and water 7 c.c. The solution works rapidly in the cold if at least 24 hours old. With a fresh solution warming or more time is necessary. It keeps 1–2 months.

[2] Kerr and Mason: *British Med. J.*, 1, 134, 1926 and Dilling: *British Med. J.*, 1, 220, 1926.

a reducing agent as well as by decreasing the solubility of the hemochromogen. Schumm[1] has cast some doubt on the specificity of this test for blood. For hemochromogen crystals see Fig. 106, page 352.

20. Preparation of Thrombin (Howell).[2]—Prepare fibrin from pig's blood according to directions given on page 354. Wash the fibrin thoroughly in water to remove hemoglobin. Squeeze out the water, mince the fibrin and cover with an 8 per cent sodium chloride solution and allow to stand in the cold for 48 hours. Filter. Precipitate the thrombin (and other proteins) from the filtrate by adding an equal volume of acetone. Filter the mixture rapidly through a number of small (25–50 c.c.) filters. Spread out filter papers and precipitate and dry rapidly in a current of cold air. Cut the dried papers into small pieces and treat with a volume of water equivalent to 66 per cent of the 8 per cent NaCl previously used. Allow to stand one-half hour and filter. Shake the filtrate with chloroform (10–15 c.c. per 100 c.c. filtrate) until on settling no opalescence is developed by heating a portion of the supernatant fluid. Decant the liquid and evaporate on watch glasses (2 c.c. to a watch glass) in a current of air. Thrombin so prepared may be kept indefinitely in a desiccator.

21. Variation in Size of Erythrocytes.—Prepare two small funnels with filter papers such as are used in quantitative analysis. Moisten each paper with physiological (isotonic) salt solution. Into one funnel introduce a small amount of defibrinated ox blood and into the other funnel allow blood to drop directly from a decapitated frog. Note that the filtrate from the ox blood is colored, whereas that from the frog blood is colorless. What deduction do you make regarding the relative size of the erythrocytes in ox and frog blood? Does either filtrate clot? Why?

II. Blood Serum

1. Coagulation Temperature.—Place 5 c.c. of undiluted serum in a test-tube and determine its temperature of coagulation according to the method described on page 169. Note the temperature at which a cloudiness occurs as well as the temperature at which coagulation is complete.

2. Precipitation by Alcohol.—To 5 c.c. of serum in a test-tube add twice the amount of 95 per cent alcohol and thoroughly mix by shaking. What is this precipitate? Make a confirmatory test. Test the alcoholic filtrate for protein. Explain the result.

3. Proteins of Blood Serum.—Place about 10 c.c. of serum in a small evaporating dish, dilute with 5 c.c. of water and heat to boiling. At the boiling-point acidify slightly with dilute acetic acid. Of what does this coagulum consist? Filter off the coagulum (reserve the filtrate) and test it as follows:

(a) Millon's Reaction.—Make the test according to directions given on page 162.

(b) Hopkins-Cole Reaction.—Make the test according to directions given on page 163.

4. Sugar in Serum.—To 5 c.c. of the neutralized filtrate from Experiment 3 add 5 drops of Benedict's solution and boil one minute. What do you conclude?

5. Detection of Sodium Chloride.—(a) Test a little of the filtrate from Experiment 3 for chlorides, by the use of nitric acid and silver nitrate. (b) Evaporate 5 c.c. of the filtrate from Experiment 3 in a watch glass on a water-bath. Examine the crystals and compare them with those reproduced in Fig. 105, p. 351.

[1] Schumm: *Z. physiol. Chem.*, 38, 1094, 1925.
[2] Howell: *Am. Jour. Physiol.*, 32, 264. 1913.

6. Separation of Serum Globulin and Serum Albumin.—Place 10 c.c. of blood serum in a small beaker and saturate with magnesium sulphate. What is this precipitate? Filter it off and acidify the filtrate slightly with acetic acid. What is this second precipitate? Filter this precipitate off and test the filtrate by the biuret test. What do you conclude?

III. Blood Plasma

1. Preparation of Oxalated Plasma.—Allow venous blood to run into a flask containing sufficient sodium oxalate to make a 0.2 per cent solution in the blood. Mix at once by rotation. Centrifuge at 3000 r. p. m. for 15 minutes. Remove the clear plasma by means of a pipette.

2. Preparation of Fibrinogen.—To 25 c.c. of oxalated plasma add an equal volume of saturated sodium chloride solution. Note the precipitation of fibrinogen. Filter off the precipitate (reserve the filtrate) and test it by a protein color test (see page 162).

3. Effect of Calcium Salts.—Place a small amount of oxalated plasma in a test-tube and add a few drops of a 2 per cent calcium chloride solution. What occurs? Explain it.

4. Preparation of Salted Plasma.—Allow arterial blood to run into an equal volume of a saturated solution of sodium sulphate or a 10 per cent solution of sodium chloride. Keep the mixture in a cool place for about 24 hours.

5. Effect of Dilution.—Place a few drops of salted plasma in a test-tube and dilute it with 10–15 volumes of water. What do you observe? Explain it.

IV. Fibrin

1. Preparation of Fibrin.—Allow blood to flow directly from the animal into a vessel and rapidly whip it by means of a bundle of twigs, a mass of strong cords, or a specially constructed beater. If a pure fibrin is desired it is not best to attempt to manipulate a large volume of blood at one time. After the fibrin has been collected it should be freed from any adhering blood clots and washed in water to remove further traces of blood. The pure product should be very light in color. It may be preserved under glycerol, dilute alcohol, or chloroform water.

2. Solubility.—Try the solubility of small shreds of freshly prepared fibrin in water, dilute acid and alkali.

3. Millon's Reaction.—Make the test according to directions given on page 163.

4. Glyoxylic Acid Reaction (Hopkins-Cole).—Make the test according to directions given on page 163.

5. Biuret Test.—Make the test according to directions given on page 164.

V. Detection of Blood in Stains on Cloth, Etc.

1. Identification of Corpuscles.—If the stain under examination is on cloth a portion should be extracted with a few drops of glycerol or physiological (0.9 per cent) sodium chloride solution. A drop of this solution should then be examined under the microscope to determine if corpuscles are present.

2. Tests on Aqueous Extract.—A second portion of the stain should be extracted with a small amount of water and the following tests made upon the aqueous extract:

(a) Hemochromogen.—Make a small amount of the extract alkaline by potassium hydroxide or sodium hydroxide, and heat until a brownish-green color

results. Cool and add a few drops of ammonium sulphide or Stokes' reagent (see page 414) and make a spectroscopic examination. Compare the spectrum with that of hemochromogen (see Absorption Spectra, Plate II). Hankin[1] has suggested a test based upon the formation of cyanhemochromogen and the microspectroscopical demonstration of the spectrum of this compound.

(b) Hemin Test.—Make this test upon a small drop of the aqueous extract according to the directions given on page 349.

(c) Guaiac Test.—Make this test on the aqueous extract according to the directions given on page 347. The guaiac solution may also be applied directly to the stain without previous extraction in the following manner: Moisten the stain with water, and after allowing it to stand several minutes, add an alcoholic solution of guaiac (strength about 1:60) and a little hydrogen peroxide or old turpentine. The customary blue color will be observed in the presence of blood.

(d) Benzidine Reaction.—Make this test according to directions given on page 348.

(e) Acid Hematin.—If the stain fails to dissolve in water extract with acid alcohol and examine the spectrum for absorption bands of acid hematin (see Absorption Spectra, Plate II).

[1] Hankin: *Brit. Med. Jour.*, p. 1261, 1906.
Sutherland and Mitra: *Biochemical Journal*, 8, 128, 1914.

CHAPTER XVIII

THE CHEMISTRY OF THE BLOOD. TISSUE ANALYSIS.

Generally speaking, the factors which influence the chemical composition of the blood in disease may be classified as physical and metabolic. The former includes those instances of retention due to alteration or destruction of permeable membranes in the excretory organs, such as the kidneys and liver. The accumulation of nitrogenous waste products in certain forms of nephritis, or the hypercholesterolemia associated with obstruction of the biliary ducts by gall stones, are examples of retention brought about by such processes. In the so-called metabolic diseases alteration in the chemistry of the blood may be induced by increased or diminished formation or utilization of the various constituents. Thus the decreased utilization of carbohydrate and the resultant hyperglycemia are the outstanding features of diabetes mellitus. The lipemia of severe diabetes is an example of the failure to utilize fat. In chronic nephritis with edema (nephrosis) the high cholesterol values are considered to be of metabolic origin. Without multiplying examples, suffice it to say that changes in the blood chemistry need not be anticipated unless some condition effecting formation, utilization or elimination is suspected. It is noteworthy, however, that the line of demarcation between these factors cannot be too sharply drawn. For example, both metabolic and mechanical factors are probably at work in renal diseases. Moreover the evidence that gall stones are the result and not the cause of hypercholesterolemia is not complete.

Concerning the formation of certain blood constituents, it may be mentioned that in disease their chemical nature may be different. The optical properties of glucose in diabetic blood are thought to differ from those of glucose in normal blood. In certain forms of thyroid disease the constitution of the thyroid hormone (thyroxin) is probably different from that of the normal secretion.

The development of micro-methods of analysis, and in particular the use of the colorimeter, have opened new fields to the investigator and provided the physician with new diagnostic tools. Of the hosts of methods that have appeared in recent years, only the most satisfactory are here described, except in those cases, such as glucose or uric acid, where the relative status of the methods is still undecided. In these instances a wider choice of methods is offered. In selecting a method, one should judge from the accuracy desired (whether clinical

COMPOSITION OF HUMAN BLOOD

Constituent	Normal range mg. per 100 c.c.[1]	Pathological conditions in which increases (unless otherwise noted) may be encountered
Total solids, per cent	19–23	Anhydremia. Low in hydremic plethora and anemia.
Total proteins (serum) per cent	6.5–8.2	See above. Low in nephritis with edema (nephrosis).
Albumin (serum) per cent	4.6–6.7	Muscular activity. Low in nephrosis.
Globulin (serum) per cent	1.2–2.3	Syphilis, pneumonia, uremia, anaphylaxis.
Fibrinogen (plasma) per cent	0.3–0.6	Pneumonia, septicemia. Low in liver diseases and cachexias.
Hemoglobin per cent (Haden)	15.6	Polycythemia. Low in primary and secondary anemia, chlorosis.
Total nitrogen, per cent	3.0–3.7	Varies chiefly with proteins (albumin globulin, hemoglobin).
Non-protein N	25–35	Nephritis, eclampsia, etc. See Urea N.
Urea N	10–15	Chronic and acute nephritis, metallic poisoning, cardiac failure, intestinal or prostatic obstruction, some infectious diseases. Relatively low in nephrosis.
Uric acid	2–3.5	Nephritis, gout, arthritis, eclampsia.
Creatinine	1–2	Nephritis.
Creatine	3–7	Terminal nephritis.
Amino-acid N	5–8	Leucemia, acute yellow atrophy of the liver severe nephritis.
Ammonia N	0.1–0.2	Terminal interstitial nephritis.
Undetermined N	4–18	Eclampsia.
Glucose	70–100	Diabetes, pregnancy, severe nephritis.
Total fatty acids	290–420	Diabetes, nephritis.
Cholesterol	150–190	Diabetes, nephritis, nephrosis, biliary obstruction, pregnancy. Low in pernicious anemia.
Lipoid phosphorus (lecithin)	12–14	Diabetes, nephritis, pregnancy. In anemia, low in plasma, high in cells.
Total acetone bodies (as acetone)	1.3–2.6	Diabetes.
Acetone + aceto-acetic acid (as acetone)	0.3–2.0	Diabetes.
β-Hydroxybutyric acid (as acetone)	0.5–3.0	Diabetes.
Bilirubin	0.1–0.25	Biliary obstruction, hemolytic anemias. Low in secondary anemia.
CO_2 capacity (plasma) vol. per cent	55–75[2]	Respiratory diseases, tetany. Low in diabetes, nephritis.
CO_2 content (arterial blood) vol. per cent.	45–55[2]	Respiratory diseases, tetany. Low in diabetes, nephritis.
CO_2 content (venous blood) vol. per cent	50–60[2]	Respiratory diseases, tetany. Low in diabetes, nephritis.
O_2 capacity vol. per cent	16–24[2]	Polycythemia, anhydremia. Low in cardiac and respiratory diseases, anemia,
O_2 content (arterial blood) vol. per cent.	15–23[2]	Polycythemia, anhydremia. Low in cardiac and respiratory diseases, anemia.
O_2 content (venous blood) vol. per cent	10–18[2]	Polycythemia, anhydremia. Low in cardiac and respiratory diseases, anemia.
Lactic acid	5–20	Exercise, eclampsia.
Phenols	1–2	Intestinal obstruction.
Chlorides as NaCl	450–500	Nephritis, cardiac conditions, prostatic obstruction, eclampsia, anemia. Low in diabetes, fever and pneumonia.
Sulphates, inorganic as S	0.5–1.0	Nephritis.
Phosphorus inorganic (plasma)	3–4	Nephritis. Low in rickets. Normal values 1–2 mg. higher in children.
Calcium (serum)	9–11	Low in infantile tetany, severe nephritis, parathyroidectomy.
Magnesium (serum)	2–3	No changes noted in disease.
Sodium (serum)	330	Low in cases of alkali deficit.
Potassium (serum)	16–22	Uremia, eclampsia.

[1] Figures express concentration in mg. per 100 c.c. of whole blood unless otherwise indicated in first column.

[2] Figures represent weighted averages of the observations of several investigators.

or research) and the time and apparatus available, which method to use.

In addition to the excellent survey of the clinical usefulness of blood chemistry in Victor C. Myer's "Practical Chemical Analysis of Blood," C. V. Mosby Co., St. Louis, 2nd Edition, 1924, the same author has reviewed the subject in *Physiological Reviews*, 4, 274, 1924, and in "Endocrinology and Metabolism," Vol. 3, D. Appleton and Co., New York, 1924.

The reader is also referred to "The Chemistry of the Blood in Clinical Medicine," by O. L. V. de Wesselow, William Wood and Co., New York, 1925.

Reviews of special phases of blood chemistry will be referred to under the separate heads.

A table giving the normal ranges of variation of the important blood constituents together with the pathological conditions where changes may be encountered, is given on page 357.

Colorimetry

Most methods of blood analysis are based on the production of colored compounds and the comparison of the depth of color obtained with that produced from known amounts of the substance undergoing analysis.

The instrument used for measuring the relative intensity or depth of any color with respect to the same color of standard intensity is called a *colorimeter*.

The standards employed may be of several types, their selection depending upon the nature and lability of the color developed in the test and upon the degree of permanence desired. Theoretically, the most satisfactory standard is one prepared from a solution of the substance to be analyzed, treated in exactly the same way as the unknown solution. By this procedure all the slight variations of temperature, time of heating or standing, etc., are equalized. It is possible, and indeed, sometimes desirable, to use an "artificial" standard whose value has been ascertained on the basis of some other analytical method, in which case permanence is the asset. Such an "artificial" standard may be a solution of one or more dyes, or a solution of inorganic chromogenic salts. An example of the former is the napthol green B standard recommended by Myers and Wardell in their cholesterol method for blood. Inorganic chlorides (iron, cobalt and nickel) are used by Karr in his urea method, in imitation of a standard nesslerized solution. When using "artificial" standard solutions it is necessary to check their equivalence frequently against known concentrations of the substance in question. Another type of "artificial" standard is the colored glass

plate. Here the advantage of permanence may sometimes be offset by an imperfect color match. The colored plates should be standardized by spectrophotometric measurement to insure uniform results in all laboratories. Wherever possible it is advisable to determine the plate equivalent in one's own laboratory as mentioned above. With practice, a plate that is slightly off shade can be used with impunity to compare intensities of color.

Beer's law, upon which colorimetry is based, states that light in passing through a colored medium, is absorbed in direct proportion to the concentration of colored substance. Thus the *transmitted* light, which is observed through a colorimeter, varies *inversely* as the concentration. In other words it would require 20 mm. depth of a one per cent solution of a dye to transmit the same intensity of color as 10 mm. of a 2 per cent solution of the dye. Stated mathematically,

$$\frac{c_1}{c_2} = \frac{R_2}{R_1}$$

in which R_1 and R_2 are the readings (depths) on the colorimeter of the unknown and standard solutions, and c_1 and c_2 their respective concentrations. This may be written

$$c_1 = \frac{R_2}{R_1} \times c_2.$$

c_1 in this equation would be the concentration in the final unknown solution. If only a small amount of material were used in the test and it was desirable to express the concentration in terms of 100 c.c., for example, the latter would be obtained by multiplying by a volume factor:

$$C_1 \text{ (concentration in 100 c.c.)} = \frac{R_2}{R_1} \times c_2 \times \frac{100}{v}$$

where v represents the actual volume of material treated in the colorimetric test. This is on the assumption that the final volumes of colorimetric solutions of the standard and unknown are the same. In some determinations, *e.g.*, creatinine, this may not be the case. A dilution factor is accordingly introduced to correct for this difference. The final *equation upon which all colorimetric calculations are based*, becomes therefore,

$$C_1 = \frac{R_2}{R_1} \times c_2 \times \frac{100}{v} \times \frac{D_1}{D_2}$$

D_1 and D_2 being respectively the dilutions of the unknown and standard colorimetric solutions. The equation is greatly simplified by choosing values of c_2, v, and colorimeter settings with that end in view. When the standard solution is set at a certain depth (R_2) the final calculation usually involves dividing the reading of the unknown into some sample

factor, which, however, cannot be done mentally. Instead of using elaborate tables or calculating devices for this purpose, the expedient, described by Stoner,[1] of setting the unknown at a given depth and reading the standard, enables one to complete the calculation mentally by a simple multiplication, such as by 2 or 5 or decimal values of such numbers. For example, in Benedict's uric acid method, setting the *standard* at 20 mm. requires a division of the reading of the unknown into 80; while setting the *unknown* at 20 mm. requires only a multiplication of the reading of the standard by 0.2. The advantage of this manipulatory procedure will be further evident in the presentation of the calculation for the various methods to be described.

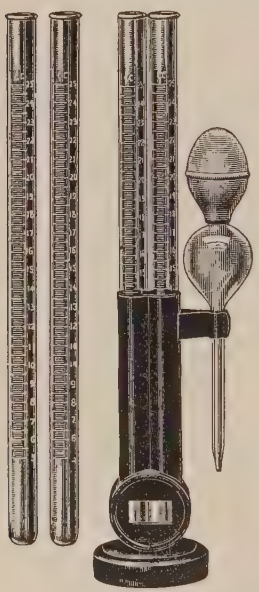


FIG. 107.—MYER'S TEST-TUBE COLORIMETER. From Myers, "Practical Chemical Analysis of Blood," St. Louis, 1924

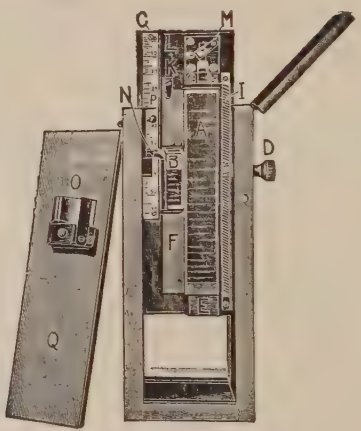


FIG. 108.—HELLIGE-LEITZ WEDGE COLORIMETER.

The choice of colorimeters is a matter of some moment. At the outset it might be stated that the least expensive instrument is not the most economical. The type of instrument selected depends on the uses to which it is to be put. The simple dilution colorimeters, such as the Sahli or Myers' (Fig. 107), are inexpensive and satisfactory where great accuracy is not expected. Colorimeters of this design may easily be constructed in the laboratory. Those instruments supplied with colored glass standards in place of standard solutions are preferable. Colorimeter blocks (Fig. 5) provided with standard tubes are widely used in phenolsulphonephthalein tests of kidney function (p. 675) and in hydrogen ion work (p. 448). The readings are made by direct compari-

[1] Stoner: *Jour. Lab. Clin. Med.*, 10, 574, 1925. See also McCrackan, Passamaneck and Harman: *J. Chem. Educ.*, 3, 416, 1926.

son and estimation. Wedge colorimeters, such as the Hellige-Leitz (Fig. 108), contain an accurately graduated wedge filled with a standard solution, the observed depth of which is adjusted by a thumb screw to match the color of an unknown solution in an adjacent cell of definite

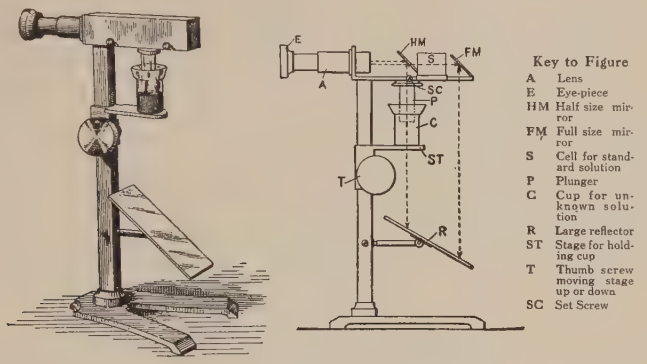


FIG. 109.—BOCK BENEDICT COLORIMETER. FROM MYERS, "PRACTICAL CHEMICAL ANALYSIS OF BLOOD," ST. LOUIS, 1924.

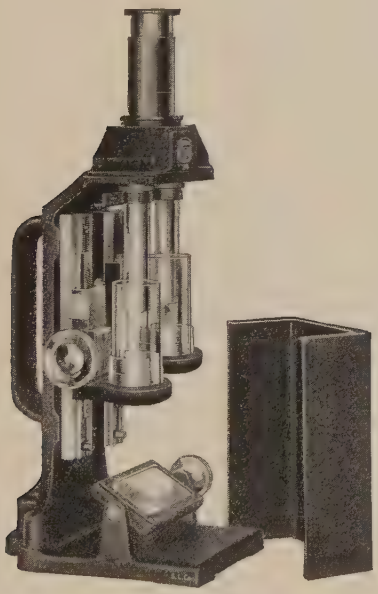


FIG. 110.—SPENCER-DUBOSCQ COLORIMETER.

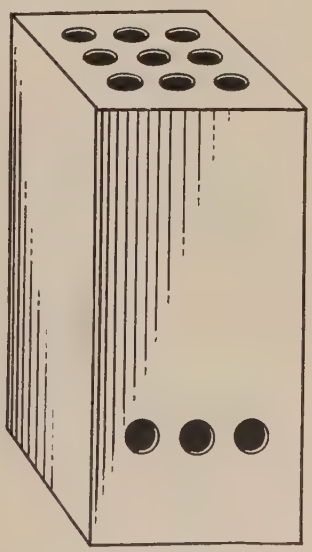


FIG. 111.—TRIPLE ROW COMPARATOR BLOCK.

thickness. The Bock-Benedict colorimeter (Fig. 109) is provided with a cup and prism for the unknown solution and a cell of definite depth for the standard solution. A system of mirrors produces a divided optical field in the eyepiece, which is evenly matched by moving the cup up or down, the depth of unknown solution being read on a vernier.

The colorimeters which are best recommended are of the Duboscq type (Fig. 110), which is provided with two prisms which dip into the solutions contained in cups, whose depth is regulated by racks and

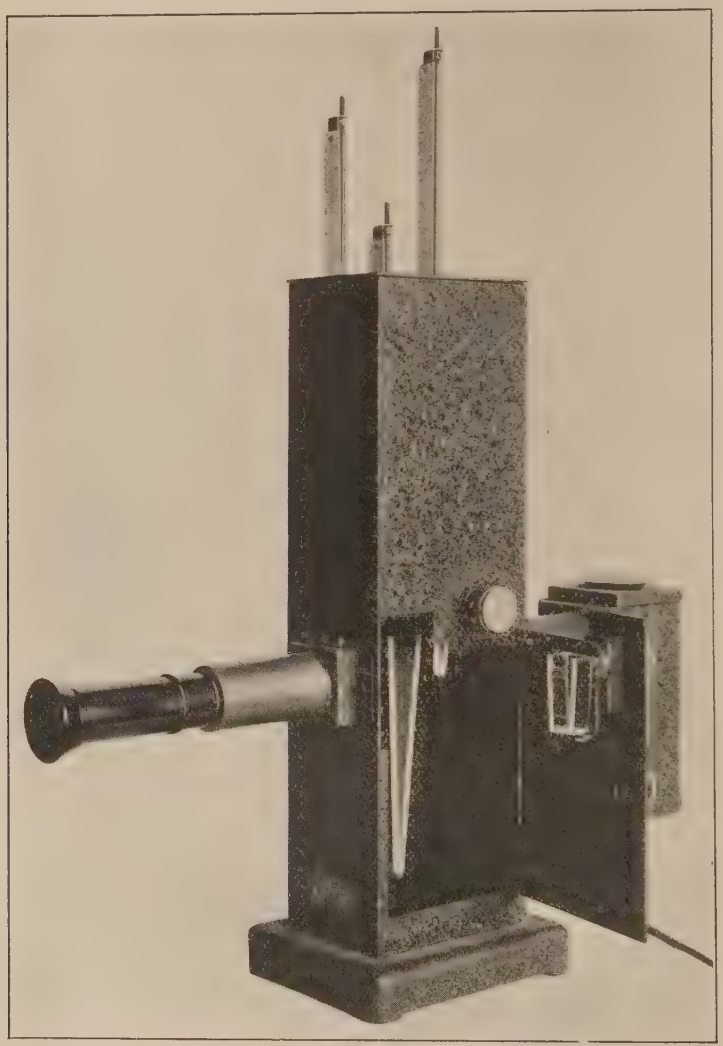


FIG. 112.—MYERS' BICOLORIMETER.

pinions. Each cup has a vernier, giving great flexibility to the use of the instrument. The divided optical field is produced by a system of prisms the optical qualities of which determine to a great extent the merit of the particular colorimeter.[1]

[1] The instruments manufactured by the Spencer Lens Co., Buffalo, N. Y., the Bausch and Lomb Optical Co., Rochester, N. Y., the Klett Manufacturing Co., New York, N. Y., and E. Leitz, New York, N. Y., are especially satisfactory in the authors' experience.

A micro colorimeter of the Duboscq type, manufactured by the Bausch and Lomb Optical Co., permits the use of smaller amounts of solution. The Buerker colorimeter made by E. Leitz is built on a somewhat different design and has a very accurate optical system.

The methods for which these colorimeters are used involve the comparison of two different intensities of the same color. In certain colorimetric analyses, however, it is necessary to measure the extent of change from one color to another. In the determination of hydrogen ion concentration, for example, indicators are used which have one color in acid solution and another in alkaline solution. For this type of work several procedures are available. Either the ordinary colorimeter block (Fig. 5) may be used with standard solutions containing the indicator in all stages of its color transformations; or a modified block (Fig. 111) consisting of three rows of holes instead of two so that sets of two standard tubes containing the acid form and the alkaline form of the indicator in varying concentrations may be used for comparison; or a bicolorimeter (Fig. 112) provided with two standard wedges containing the acid and alkaline forms of indicator may be employed. Methods in which these devices are used are discussed under the determination of the pH of blood, p. 450.

Nephelometry

The Nephelometer.—The nephelometer is an instrument for measuring the density of precipitates and thus determining the amount of any substance which can be obtained in the form of a suitable suspension. It is somewhat similar in form and principle to a colorimeter. It differs from the latter in that the light which reaches the eye is not transmitted light, which, on the contrary, is excluded, but light reflected from the particles of the suspension. The brightness of the two fields is compared instead of their colors. It is adapted particularly for the determination of substances that in very dilute solution may be precipitated in the form of suspensions which do not agglutinate appreciably in the time required for making readings (10–20 minutes). The method has been adapted to the determination of proteins in digestion mixtures, milk, urine, etc.;[1] nucleic acids;[2] chlorides,[3] phosphates, and phosphatides in blood, etc.;[4] fats in milk, blood, etc.;[5] acetone bodies in urine

[1] Kober: *Jour. Biol. Chem.*, 13, 485, 1913; *Jour. Am. Ch. Soc.*, 35, 1585, 1913; Folin and Denis: *Jour. Biol. Chem.*, 18, 273, 1914.
[2] Kober and Graves: *Jour. Am. Chem. Soc.*, 36, 1304, 1914.
[3] Richards: *Zeitschr. f. anorg. Chem.*, 7, 269, 1895.
[4] Greenwald: *Jour. Biol. Chem.*, 21, 29, 1915; Bloor: *Jour. Biol. Chem.*, 22, 133, 1915; Kober and Egerer: *Jour. Am. Chem. Soc.*, 37, 2373, 1915.
[5] Bloor: *Jour. Biol. Chem.*, 17, 377, 1914; *J. Am. Chem. Soc.*, 36, 1300, 1914.

and blood;[1] uric acid and purine bases;[2] ammonia;[3] calcium;[4] silver, etc., and is continually finding new applications. It is possible to determine very minute amounts of substances, entirely outside of the range of gravimetric methods of analysis, and hence the procedure may be used where the amount of material is very limited.

The Duboscq colorimeter has been adapted for nephelometric purposes by Kober[5] and by Bloor.[6] Bloor's nephelometer is illustrated in Figs. 113 and 114. The plungers of the colorimeter are replaced by optically clear test tubes of uniform diameter (*B*), in which are contained the suspensions to be matched. In place of colorimeter cups are opaque jackets (*C*) which are supported by means of collars (*D*). The light comes from in front of the tubes and is in part reflected upward, the intensity depending on the reflecting surface of the particles in suspension, and hence, other things being equal, on their number. The intensity of the field as viewed from above may be varied by raising or lowering the jackets, thus regulating the amount of light which reaches the suspended particles.

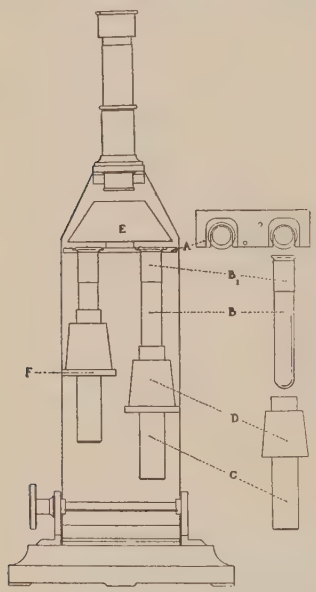


FIG. 113.—BLOOR'S NEPHELOMETER.

A—Supporting plate for
B—Nephelometer tubes.
C—Opaque jackets supported by
D—Collars, on
F—Cup supports.
E—Prism case.

Artificial light is necessary and the lamp should be enclosed in a tight box into one end of which the nephelometer fits snugly. A partition extending part way up the box as shown in the diagram (Fig. 114) serves the double purpose of shutting off the light from the lower part of the instrument and of providing a stop against which the instrument is pushed, so that its distance from the light is kept constant.

The change from colorimeter to nephelometer can be made in one or two minutes, since it consists essentially only in unscrewing the plate carrying the plungers and screwing on the plate to carry the nephelometer tubes. The extra parts needed, plate, tubes, and jackets, are few

[1] Folin and Denis: *Jour. Biol. Chem.*, 18, 263, 1914; Marriott: *Ibid.*, 16, 289, 1913.
[2] Graves and Kober: *Jour. Am. Chem. Soc.*, 37, 2430, 1915.
[3] Graves: *J. Am. Chem. Soc.*, 37, 1181, 1915.
[4] Lyman: *Jour. Biol. Chem.*, 21, 551, 1915.
[5] Kober: *Jour. Biol. Chem.*, 13, 485, 1913; *Jour. Am. Chem. Soc.*, 35, 1585, 1913.
[6] Bloor: *Jour. Biol. Chem.*, 22, 145, 1915.

and can be made if necessary from material at hand in any laboratory and by anyone with a slight degree of mechanical skill.[1]

A still simpler form of nephelometer is constructed from a colorimeter by placing opaque jackets of some inert material around the plungers, covering all but their under surfaces. The suspensions are contained in glass cups which have opaque (metal) bottoms. The only light that reaches the eye therefore, is that which comes from in front of the cups and is reflected upward through the plungers.

The above description applies only to the later type of colorimeter where the cups move and the prisms are stationary. The changes required to convert the older type of instrument are more complicated

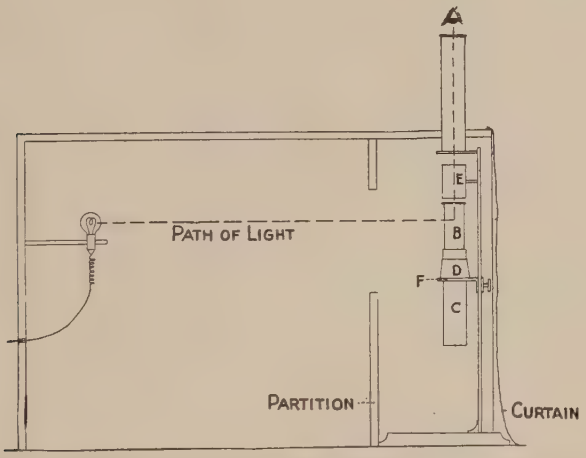


FIG. 114.—NEPHELOMETER IN POSITION, SHOWING RELATION TO SOURCE OF LIGHT.

and scarcely to be advised unless the instrument is to have fairly continuous use as a nephelometer.

Nephelometric Calculations.—The amount of precipitate in solutions examined nephelometrically is not exactly inversely proportional to the readings of the scale. When the concentration of the unknown and of the standard are within 10 per cent of each other (or within about 20 per cent if the readings are made at depths as great as 50–60 mm.) accurate results may however be obtained directly. If the variations are greater than this a correction is necessary. Kober[2] has proposed an equation to supply this correction and thus make possible more accurate work under conditions of moderate variations of concentration.

[1] The extra parts necessary for the conversion of the colorimeter into the nephelometer may be obtained from the International Instrument Co. of Cambridge, Mass.

[2] Kober: *J. Am. Chem. Soc.*, 37, 2379, 1915; *Jour. Biol. Chem.*, 13, 485, 1913

Methods[1]

The Drawing of Blood for Analysis.—A tourniquet (of soft, firm rubber tubing or a strip of bandage) is drawn tightly about the arm of the patient a couple of inches above the elbow. The fist is kept firmly clenched. The parts about the most prominent vein (usually the median basilic) are washed with alcohol, the vein is held immobile by the thumb of the operator, and a sterile hypodermic needle, sharp but without too long a point, (a No. 18, an inch and a half long may be used), inserted into the vein, at an angle of about 50° with the surface of the arm, the opening of the needle being kept upward or to the side. Blood is allowed to flow into a test tube the inner wall of which is coated with a film of sodium oxalate. Sufficient oxalate is dissolved by the blood to prevent clotting.[2] Plasma may be obtained by centrifugation. For special precautions to be used in drawing blood for the determination of the carbon dioxide content of plasma or oxygen content of whole blood, see pp. 434 and 451.

Blood specimens are best taken in the morning before breakfast, to minimize the influence of food ingestion. Specimens should be kept in the ice box and analyses preferably made on the day of withdrawal. This is particularly necessary in the case of sugar, which decreases in amount on standing. Denis[3] has shown, however, that at least for the Folin and Wu sugar method blood may be preserved for four days or more at 20–33°C., if one drop (1 30 c.c.) of commercial formalin (40 per cent) solution is added to each 5 c.c. of blood. Fluorides alone[4] or with thymol[5] have been recommended as preservatives, but care must be exercised in their selection inasmuch as they all interfere with certain methods. The Folin-Wu filtrate will keep over night better than whole blood. If the blood is to be transported for blood sugar estimation, a picric acid method (see p. 387) may be used, since this precipitant also preserves the specimen.

By the Folin-Wu[6] system we may determine on a single blood filtrate the following: Non-protein nitrogen, urea, uric acid, creatinine and creatine, sugar, amino-acids, and chlorides. About 10 c.c. of blood are needed for the combined determinations.

1. **Preparation of the Protein-free Blood Filtrate.**—*Principle.*—The total proteins of the blood are removed by precipitation with tungstic acid (formed by the interaction of sodium tungstate and sulphuric acid) and filtration. The filtrate contains all of the constituents of the blood determined by this system.

[1] Methods for the analysis of blood gases and hemoglobin and for hydrogen ion concentration are given in the chapter on "Respiratory Metabolism and Neutrality Regulation," p. 419.

[2] 0.5 c.c. of hot saturated (20%) sodium oxalate is placed in test tubes which are then laid flat on a hot plate. The evaporating solution spatters and, drying, forms a coating of oxalate on the wall of the tube.

[3] Denis, W.: *Jour. Biol. Chem.*, 44, 203, 1920.

[4] Denis and Bevon: *J. Lab. Clin. Med.*, 9, 674, 1924.
Major, *J. Am. Med. Assn.*, 83, 1952, 1923.

[5] Sander: *Jour. Biol. Chem.*, 58, 1, 1923. John, *Arch. Path. Lab. Med.*, 1, 227, 1926, after an extensive study of blood preservatives recommends sodium fluoride and thymol in the proportion of 20:1, using 20 mg. of the mixture per 10 c.c. of blood.

[6] Folin and Wu: *Jour. Biol. Chem.*, 38, 81, 1919.

Procedure.—To prevent coagulation 20 mgs. of sodium or potassium oxalate per 10 c.c. of blood should have been used. Use of much larger amounts of oxalate or the use of citrate interferes with deproteinization, and interferes more or less with the uric acid determination.

Transfer a measured quantity (5 to 15 c.c.) of oxalated blood to a flask having a capacity of fifteen to twenty times that of the volume taken. Lake the blood with seven volumes of water. Add one volume of 10 per cent solution of sodium tungstate[1] ($Na_2WO_4 2H_2O$) and mix.

Add from a graduated pipette or burette, very slowly (drop by drop) and with constant shaking, one volume of two-thirds normal sulphuric acid.[2] Close the mouth of the flask with a rubber stopper and shake. If the conditions are right, hardly a single air bubble will form as a result of the shaking. Let stand for 5 minutes (10 to 20 minutes if uric acid is to be determined). The color of the coagulum gradually changes from bright red to dark brown. If this change in color does not occur, the coagulation is incomplete, usually because too much oxalate is present. In such an emergency the sample may be saved by adding 10 per cent sulphuric acid, one drop at a time shaking vigorously after each drop, and continuing until there is practically no foaming and until the dark brown color has set in.

Pour the mixture on a filter large enough to hold it all. This filtration should be begun by adding only a few c.c. of the mixture down the double portion of the filter paper and withholding the remainder until the whole filter has been wet. Then the whole of the mixture is poured on the funnel and covered with a watch glass. If the filtration is made as described the very first portion of the filtrate should be clear as water and no re-filtering is necessary.

An improved method for preparing the protein-free filtrate has been introduced by Haden.[3] The advantages claimed are the use of fewer solutions, more rapid filtration, increased quantity of filtrate and more neutral filtrate. The modification consists in combining the seven volumes of water with the sulphuric acid, making 8 volumes of $\frac{N}{12}$ acid to be used. Eight volumes of $\frac{N}{12}$ sulphuric

FIG. 115. DILUTING PIPETTE. (Folin and Wu: *Jour. Biol. Chem.*, 38, 81, 1919).

[1] A 10 per cent solution of sodium tungstate. Some sodium tungstates, though labeled c.p., are not serviceable for this work. They usually contain too much sodium carbonate. The c.p. sodium tungstate made by the J. T. Baker Chemical Company, Phillipsburg, N. J., is satisfactory.

[2] A two-thirds normal sulphuric acid solution, 35 g. of concentrated c.p. sulphuric acid diluted to a volume of 1 liter, will usually be found to be correct; but it is advisable, indeed necessary, to check it up by titration. The two-thirds normal acid is intended to be equivalent to the sodium content of the tungstate so that when equal volumes are mixed substantially the whole of the tungstic acid is set free without the presence of an excess of sulphuric acid. The tungstic acid set free is nearly quantitatively taken up by the proteins and the blood filtrates obtained are, therefore, only slightly acid to congo red paper. If plasma is used half amounts of sodium tungstate and sulphuric acid solutions should be taken, the balance being made up with water.

[3] Haden: *Jour. Biol. Chem.*, 56, 469, 1923.

acid[1] are added directly to the blood, whereupon laking and darkening occur very rapidly. One volume of 10 per cent sodium tungstate solution is then added, the mixture well shaken and filtered as above described.

Special graduated "blood pipettes," are very useful for the measurement of the blood, the tungstate and the acid. (See Fig. 115.)

The protein-free blood filtrates are not acid enough to prevent bacterial decomposition. If the filtrates are to be kept for any length of time, more than two days, some preservative, a few drops of toluene or xylene, should be added.

For optimum precipitation of protein, the filtrate should not be more alkaline than pH 2.8.[2] A drop of 0.04 per cent bromphenol blue added to a few drops of filtrate on a test plate should give a yellow or greenish-yellow color, alkalinity being denoted by a pure blue shade.

2. Determination of Non-protein Nitrogen.—*Principle.*—Nitrogen is determined in a portion of the blood filtrate by a micro-Kjeldahl method, using a sulphuric and phosphoric acid mixture for the digestion,[3] the ammonia formed being determined colorimetrically after direct nesslerization of the digestion mixture.

Procedure.—Transfer 5 c.c. of the blood filtrate to a large test tube (Pyrex) 200 mm. × 25 mm., preferably graduated at 35 c.c. and 50 c.c. The test tube should either be dry or rinsed with alcohol to reduce the danger of bumping. Add 1 c.c. of diluted acid mixture[4] and a quartz pebble. Boil vigorously over a micro burner until the characteristic dense fumes begin to fill the tube. This will happen in from 3 to 7 minutes, depending on the size of the flame. When the test tube is nearly full of fumes reduce the flame sharply so that the speed of the boiling is reduced almost to the vanishing point. Cover the mouth of the test tube with a watch glass. Continue the gentle heating for 2 minutes, counting from the time the test tube became filled with fumes. If the oxidations are not visibly finished at the end of two minutes the heating must be continued until the solution is nearly colorless. Usually the solution becomes colorless at the end of 20 to 40 seconds. At the end of 2 minutes remove the flame and allow the digestion mixture to cool for 70 to 90 seconds.[5] Then add 15 to 25 c.c. of

[1] Add 2.5 c.c. concentrated sulphuric acid to 1 liter distilled water. Mix well and check by titration against 0.1 N NaOH. 20 c.c. of $\frac{N}{12}$ acid should require 16.7 c.c. $\frac{N}{10}$ NaOH for its neutralization.

[2] Merrill: *Jour. Biol. Chem.*, 60, 257, 1924.

[3] The proposed modifications of this method consist chiefly in varying the digestion mixture. Hydrogen peroxide has been found very effective (see Method 2A below) and was used by Prof. V. C. Myers before it was described in the literature.

[4] Made by diluting regular acid mixture with an equal volume of water. The regular acid mixture is made as follows: To 50 c.c. of a 5 per cent copper sulphate solution add 300 c.c. of 85 per cent phosphoric acid and mix. Add 100 c.c. of concentrated sulphuric acid free from the least trace of ammonia and mix. Keep well protected to prevent absorption of ammonia from the air.

[5] McCracken, Passamaneck and Harman (*J. Lab. Clin. Med.*, 11, 678, 1926) recommend, and describe apparatus for, distillation with sodium hydroxide preliminary to nesslerization.

water. Cool further approximately to room temperature and then fill to the 35 c.c. mark with water. Add 15 c.c. of Nessler's solution (see section on Reagents and Solutions, Appendix). Insert a clean rubber stopper and mix. If the solution is turbid, centrifuge a portion before making the color comparison with the standard.

The standard most commonly required is 0.3 mg. of N. Add 3 c.c. of standard ammonium sulphate solution (containing 1 mg. of N per 10 c.c., made by dissolving 0.4716 gm. of specially purified ammonium sulphate (see note p. 715) in one liter of ammonia-free distilled water) to a 100 c.c. volumetric flask. Add to it 2 c.c. of the diluted phosphoric sulphuric acid mixture, to balance the acid in the test tube; dilute to about 60 c.c., add 30 c.c. of Nessler's solution and then dilute to the mark. The unknown and the standard should be nesslerized simultaneously.

Calculation.—If the standard is set at 20 mm. for the color comparison, 20 divided by the reading and multiplied by 0.3 gives the non-protein nitrogen in 1 c.c. of blood, because 0.5 c.c. (the amount of blood represented in 5 c.c. of the blood filtrate) nesslerized at a volume of 50 c.c. is equivalent to 1 c.c. nesslerized at a volume of 100 c.c. The non-protein nitrogen per 100 c.c. of blood is, therefore, 20 divided by the reading and multiplied by 30 (0.3 times 100).

The calculation may be eliminated entirely if the unknown is set at 30 mm., in which case the reading of the standard equals the mg. non-protein N per 100 c.c.

Alternate Procedures.—Instead of nesslerizing, it is possible to distill or aspirate off the ammonia into standard acid, and titrate using apparatus of the types used in the micro-determinations of total nitrogen in urine (see Chapter XXXI). Stehle has suggested[1] a gasometric method along the line of his method for urea in urine (see Chapter XXXI).

Interpretation.[2]—Normal blood contains 25–35 mg. of non-protein nitrogen per 100 c.c. In early interstitial nephritis values of 30–50 may be obtained, and in severe nephritis much higher values, up to the 400 mg. occasionally found in uremia. Non-protein nitrogen, being a collective expression, varies with its constituents, particularly urea (*q.v.*).

The non-protein nitrogen of the blood includes nitrogen present in urea, uric acid, creatinine, ammonia, amino-acids and other substances. The "rest N" is of undetermined composition and constitutes almost half of the total non-protein nitrogen of whole blood. It is contained chiefly in the corpuscles. In uremia the proportion of nitrogen in his form falls to much lower levels.

In regard to the choice of determining either the non-protein or the urea N as a clinical test of renal function, the advantages seem to be in favor of the latter, for the following reasons: urea covers a relatively

[1] *Jour. Biol. Chem.*, 45, 223, 1920. According to the author, the pyrogallate treatment may be dispensed with if copper sulphate is omitted.

[2] For review, see "Non-protein Nitrogen of Blood in Health and Disease," by Otto Folin, *Physiol. Rev.* 2, 460, 1922.

wider range of variation in disease; it is a single chemical substance rather than a group of substances some of whose composition is undetermined; it is simple to determine clinically, especially if the direct nesslerization of the Folin-Wu filtrate is employed. It is of interest, however, that the urea fraction of the non-protein nitrogen, which usually rises in renal disease, shows subnormal values in cases of eclampsia, suggesting an increase in the rest N fraction which may contain toxic split-products of protein metabolism.[1]

2A. Koch-McMeekin Method.[2] **Principle.**—In this method sulphuric acid and hydrogen peroxide are used in the digestion.

Procedure.—Transfer 5 c.c. of the blood filtrate to a 200 × 25 mm. Pyrex test tube, add 1 c.c. of 1 : 1 sulphuric acid and evaporate off the water by heating over free flame (or sand bath) with shaking. Heat over a micro-burner until dense white fumes fill the tube. Then add 1 to 3 drops of 30 per cent hydrogen peroxide allowing it to drop directly into the solution. Heat again to boiling. If the solution discolors repeat addition of peroxide and finally boil gently for 5 minutes. Cool, transfer to a 50 c.c. volumetric flask with about 35 c.c. of water and add with rotation 12 c.c. of modified Nessler solution.[3] Dilute to 50 c.c. and mix. It is also possible to use tubes graduated at 35 and 50 c.c. throughout the procedure as in preceding method. The standard consists of 0.1 to 0.3 (usually 0.3) mg. of nitrogen (1 to 3 c.c. of standard ammonium sulphate solution) diluted to 35 c.c. and nesslerized as above. After 5–20 minutes compare in a colorimeter.

Calculation.—The calculation is the same as in the preceding method.

3. Determination of Urea.—*Principle.*—The urea is decomposed to ammonium carbonate by means of the enzyme urease, in the presence of phosphate, which maintains suitable reaction in the mixture and also catalyzes the conversion. The ammonia is distilled off and determined colorimetrically after nesslerization. Alternate aeration and autoclave procedures are given.

Procedure.—Transfer 5 c.c. of the tungstic acid blood filtrate to a Pyrex ignition tube (200 × 25 mm.) This test tube must be rinsed with nitric acid and then with water if it has contained Nessler solution. Add 2 drops of buffer mixture[4] and then introduce 1 c.c. of urease solution.[5] Immerse the test tube in warm water, 40 to 55°C., and leave it there for 5 minutes, or let stand at room temperature for 15 minutes.

The ammonia formed from the urea is most conveniently obtained by distil-

[1] Killian and Sherwin: *Amer. Jour. Obst. and Gyn.*, 2, 6, 1921.

[2] Koch and McMeekin: *J. Am. Chem. Soc.*, 56, 2066, 1924. A stronger acid digestion mixture, containing perchloric acid is used by Rose, *Jour. Biol. Chem.*, 64, 253, 1925.

[3] For method of preparation of Nessler solution and other details see method for urine p. 717. If the peroxide contains nitrogen a correction must be made for this.

[4] Made by dissolving 69 gm. of monosodium phosphate and 179 gm. of crystallized disodium phosphate in 800 c.c. of warm distilled water and diluting to one liter.

[5] Urease solution. Wash about 3 gm. of permutit in a flask, once with 2 per cent acetic acid, then twice with water; add 5 gm. of fine jack bean meal and 100 c.c. of 15 per cent alcohol. Shake gently but continuously for 10–15 minutes, pour on large filter, and cover with a watch glass. The solution may be kept about a week at room temperature or 4–6 weeks in an ice box.

lation, without a condenser, and using a test tube graduated at 25 c.c. and containing 2 c.c. of 0.05 N hydrochloric acid as the receiver. The illustration shows a compact and convenient arrangement for this distillation.[1]

Add to the urease blood filtrate a dry pebble, a drop or two of paraffin oil and 2 c.c. of saturated borax solution. Insert firmly the rubber stopper carrying the delivery tube and receiver and then boil at a moderately fast, uniform rate for 4 minutes. The size of the flame should never be cut down during the distillation, nor should the boiling be so brisk that the emission of steam from the receiver begins before the end of 3 minutes. At the end of 4 minutes slip off the receiver from the rubber stopper and let it rest in a slanting position while the distillation is continued for 1 more minute. Rinse the lower end of the delivery tube with a little water and cool the distillate with running water and dilute to about 20 c.c. Transfer 0.3 mg. N (3 c.c. of the standard ammonium sulphate solution) to a 100 c.c. volumetric flask and dilute to about 75 c.c. Nesslerize, using 10 c.c. of Nessler's solution for the standard, and 2.5 c.c. for the unknown in the test tube. Dilute both to volume and make the color comparison.

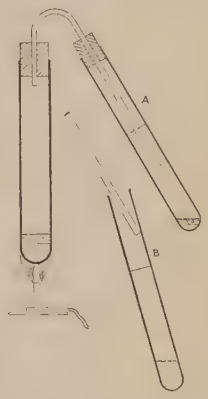


FIG. 116.—APPARATUS FOR DISTILLATION OF AMMONIA FROM UREA. A, FIRST POSITION. B, SECOND POSITION.

(Folin and Wu: *Jour. Biol. Chem.*, 38, 81, 1919.) A groove may be cut along the side of the stopper of the receiving tube to permit the escape of steam.

Calculation.—Divide 20 (the height of the standard in mm.) by the colorimetric reading and multiply by 15. This gives the urea nitrogen in mg. per 100 c.c. of blood. In explanation of this calculation it is to be noted that the unknown representing 0.5 c.c. of blood, is nesslerized at 25 c.c., whereas in the case of the non-protein nitrogen it is nesslerized at a volume of 50 c.c. The same colorimetric reading, therefore, represents only one-half as much nitrogen in the urea determination as in the non-protein nitrogen determination.

By setting the unknown at 15, the reading of the standard gives directly the mg. urea N per 100 c.c. of blood, thus eliminating further calculation.

Urea Determination by Means of the Autoclave.—When a large number of urea determinations are to be made or when creatin determinations are also made, it is sometimes convenient to decompose the urea of the blood filtrate by heating under pressure. To 5 c.c. of the blood filtrate in a large test tube add 1 c.c. of normal hydrochloric acid, cover with tin foil and heat to 150° for 10 minutes. Distill off the ammonia exactly as in the preceding process, except that 2 c.c. of 10 per cent sodium carbonate must be substituted for the borax, because of the added hydrochloric acid.

Aeration Process in Urea Determination.—The ammonia formed from the blood urea by urease, or by heating under pressure, can, of course be, driven into the receiver by an air current plus an alkali, instead of by the distillation process described above. The aeration process gives perfectly reliable results, if a good air current is available.

To the decomposed blood filtrate in a large test tube add a little paraffin oil and 1 or 2 c.c. of 10 per cent sodium hydroxide. Connect with a smaller test tube,

[1] Watson and White have suggested a modification of this apparatus. See *Jour. Biol. Chem.*, 45, 465, 1921.

marked at 25 c.c., and containing 2 c.c. of 0.5 N hydrochloric acid. The connection is made as in the aeration process for urea in urine. Pass the air current through rather slowly for 1 minute and then nearly as fast as the apparatus can stand for 10 to 15 minutes. Rinse the connecting tube; dilute the contents of the receiver to 20 c.c., add 2.5 c.c. of Nessler solution, dilute to the 25 c.c. mark, and make the color comparison in the usual manner.

Interpretation.[1]—Normally, the urea nitrogen of blood varies between 10 and 15 mg. per 100 c.c. On the restricted hospital diet, however, values below 20 mg. should not be regarded as abnormal. In early nephritis the urea nitrogen may rise to 30 or 40 mg., but in the terminal stages of chronic nephritis and in some cases of acute nephritis

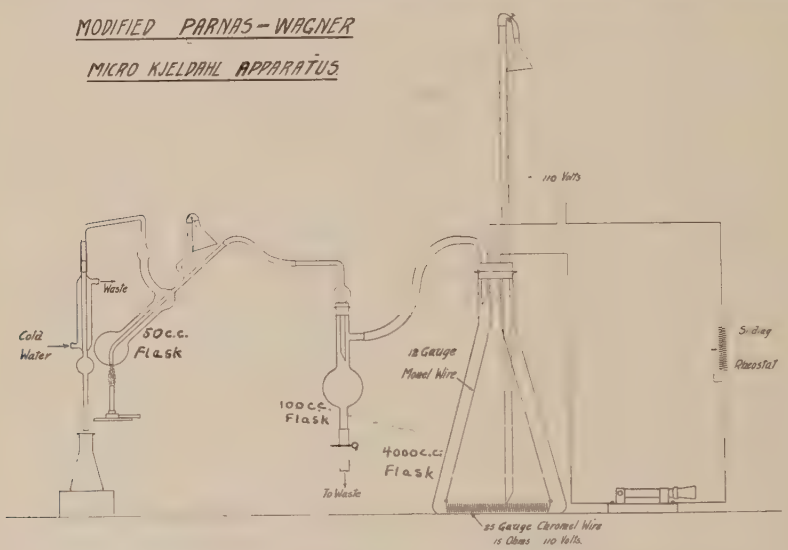


Fig. 117.—Distillation Apparatus Used in Clark-Collip Urea Method.

marked urea retention may occur. High values may also be found in other conditions associated with damaged renal function, such as bichloride poisoning, double polycystic kidney, intestinal obstruction, prostatic obstruction (in which case the urea N constitutes a valuable guide to surgical risk), lead poisoning, certain infections, cardiac failure, and so on. Relatively low figures for urea nitrogen are found in nephrosis (tubular nephritis with edema), which is probably of metabolic rather than renal origin.

3A. Clark-Collip Autoclave Method.[2] **Procedure.—To 5 c.c. of blood filtrate in a lipped test tube (25 × 120 mm.) add 1 c.c. of N HCl, cover with tinfoil and autoclave at 150° for 10 minutes. Transfer to the distilling apparatus (see Fig. 117) add 3 c.c. of N NaOH and distill into 0.01 N HCl. Titrate excess acid with**

[1] Folin: *Physiol. Rev.*, 2, 460, 1922.
Folin and Berglund: *Jour. Biol. Chem.*, 51, 395, 1922.
[2] Clark and Collip: *Jour. Biol. Chem.*, 67, 679, 1926 and private communication.

0.01 N NaOH using methyl red as an indicator following strictly the technique of Pregl.[1] Mg. urea per 100 c.c. blood = 28 × c.c. of 0.01 N acid used.

3B. Karr's Method for the Determination of Blood Urea by Direct Nesslerization of Folin-Wu Filtrate.[2] **Principle.**—By the action of urease, the urea in the filtrate is converted to ammonium carbonate which is nesslerized and compared colorimetrically with a standard urea solution similarly treated. The interference of peptones and amino acids (Folin and Wu) is regarded as too slight and invariable to influence the clinical value of the data obtained. The advantages of this method are speed and practicability of performing many determinations at one time, both important factors in routine practice.

Procedure.—Five c.c. of the Folin-Wu blood filtrate are transferred to a test tube followed by one drop of buffer solution[3] and either a strip of urease paper[4] or 5 drops of urease solution.[5] In another test tube, 5 c.c. of the standard urea solution[6] are similarly treated. The tubes are placed in a bath at 50°C. for 15 minutes. The contents of the conversion tubes are then transferred with rinsings to test tubes graduated at 22.5 and 25 c.c.[7] and diluted to the 22.5 c.c. mark. Nessler's solution is added to each tube up to the 25 c.c. mark and colorimetric readings are made about one minute later.

Blood filtrates high in urea N (35 mg. or over, per 100 c.c.) give a cloudy solution on nesslerization, in which case the determination is repeated, using smaller amounts of filtrate.

In laboratories where many determinations of blood urea N are conducted daily it is advisable to use an artificial permanent standard (see original paper) which is controlled by daily checking against the standard urea solution.

Calculation.—Set the unknown on the left at 15 mm. The reading of the standard then gives directly the urea N per 100 c.c. of blood. If less than 5 c.c. of filtrate (0.5 c.c. blood) were used make the necessary correction.

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times .075 \times \frac{100}{.5} = \text{mg. Urea N}$$

per 100 c.c. of blood.

[1] Pregl: Quantitative Organic Microanalysis. Translated by Fyleman, Blakiston's Son and Co., Philadelphia, 1924.

[2] Karr: *J. Lab. Clin. Med.*, 9, 3, 1924. Roe and Irish (*Ibid.* 11, 1087, 1926) remove traces of protein by adsorption on $Ca_3(PO_4)_2$, thus minimizing turbidity.

[3] Prepared by dissolving "14 gm. of sodium pyrophosphate [$Na_4P_2O_7 \cdot 10H_2O$] in enough half normal phosphoric acid to make 100 c.c. The half normal phosphoric acid is made by diluting 20 c.c. of 85 per cent phosphoric acid to one liter and titrating 5 c.c. with tenth normal alkali and phenolphthalein as indicator, to a faint pink color. On the basis of this titration, dilute the acid to a substantially correct half normal solution."

[4] Prepared according to Folin's directions. Shake 15 gm. of Jack bean meal with 5 gm. of Permutit in 100 c.c. of 15 per cent alcohol for 15 minutes by means of a mechanical shaker or for one-half hour by hand. Filter through a fluted filter paper, preferably in the ice-box over night. Transfer the filtrate to an open dish and draw pieces of heavy-ammonia-free filter paper through it and hang them up to dry. When dry cut into strips about 1 by 1½ inches.

[5] Prepared as directed for urease paper. The solution is not as stable as the dried paper and should be made fresh about every other week and kept on ice. If determinations are run only occasionally, the enzyme paper is preferable.

[6] Prepared by diluting 5 c.c. of a stock urea solution to 100 c.c. with water (5 c.c. = 0.075 mg. N). The stock urea solution contains 0.1286 gm. urea in 200 c.c.

[7] Separate tubes for nesslerization are necessary because of the "poisoning" action of mercury on urease. When this is suspected in the conversion tubes they should be cleaned with strong nitric acid. Instead of graduated test tubes, ordinary 25 c.c. graduated cylinders may be used.

3C. Direct Aeration Method. (Colorimetric Procedure of Myers[1]). Principle.—This method is based on those of Folin and Denis,[2] Marshall,[3] and Van Slyke and Cullen;[4] The urea of the blood is converted to ammonium carbonate by the action of urease, the ammonia aerated off into an acidified solution and determined by nesslerization. The same apparatus (Fig. 118) may be employed for the estimation of urea in urine.

Procedure.—Into a lipless test tube just small enough to fit into a 100 c.c. ungraduated cylinder, are introduced 1 c.c. of urease solution,[5] and 2 drops of the activating phosphate solution[6] if this is not already present. Two c.c. of the oxalated blood are now added, preferably with an Ostwald-Folin pipette, and the tube incubated at 50°C. for 15 minutes.

Into a 100 c.c. graduated cylinder without lip are placed 15 c.c. of distilled water and 2 or 3 drops of 10 per cent HCl. This is now closed with a 2-holed stopper provided with a glass tube passing nearly to the bottom of the cylinder.

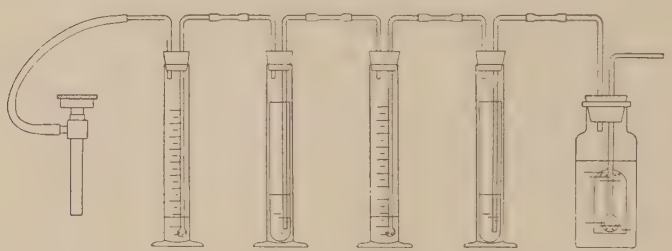


FIG. 118.—ASPIRATION APPARATUS FOR UREA DETERMINATION.

(Myers: "Practical Chemical Analysis of the Blood," C. V. Mosby Co., St. Louis, 1924. Myers suggests the use of test tubes within the cylinders as illustrated for ease in manipulation.)

The test tube containing the digested mixture is placed in the ungraduated cylinder, and the connecting tubes adjusted. Five drops of caprylic alcohol and 4 or 5 c.c. of saturated (20 per cent) sodium carbonate are allowed to run down the side of the tube containing the blood digest, and the stopper quickly inserted. The cylinder containing the digested mixture is connected with a wash bottle containing dilute sulphuric acid (to remove NH_3 from the incoming air). The outlet tube of the graduated cylinder is now connected with a suction pump and air slowly allowed to pass through the apparatus, the speed being increased so that at the end of two minutes the air current is as rapid as the apparatus will stand. Aeration is complete in about 30 minutes and the apparatus is then disconnected.

5 c.c. of standard ammonium sulphate solution[7] are introduced into a 100 c.c. volumetric flask and 50 to 60 c.c. of distilled water are added. 20 c.c. of Nessler's

[1] Myers: "Practical Chemical Analysis of the Blood," C. V. Mosby Co., St. Louis, 2nd edition, 1924.

[2] Folin and Denis: *Jour. Biol. Chem.*, 11, 527, 1912.

[3] Marshall: *Jour. Biol. Chem.*, 15, 487, 1913.

[4] Van Slyke and Cullen. *Jour. Biol. Chem.*, 19, 211, 1914.

[5] Concentrated preparations of jack bean urease may be obtained on the market (Arlington Chemical Co., Yonkers, N. Y.), prepared according to the formula of Van Slyke and Cullen, and containing the activating phosphate. The preparation described on p. 720 may be used.

[6] Folin-Wu preparation. See footnote 3, p. 373.

[7] Dissolve 0.944 gm. ammonium sulphate (pyridine-free) in distilled water and dilute to 1000 c.c. 5 c.c. of this solution contain 1 mg. N.

solution (Appendix) are added, followed by distilled water to the 100 c.c. mark. At the same time, 7 to 8 c.c. of Nessler's solution are added to the unknown and the volume made up to 25 c.c. in the graduate. The solutions are then compared in the colorimeter. For high concentrations of urea N, increase the amount of Nessler's solution and the dilution until the color approximates the standard.

Calculation.

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 1 \times \frac{100}{2} \times \frac{25}{100} = \text{mg. urea N per 100 c.c. blood.}$$

Set the unknown on the left at 12.5 mm. and the reading of the standard gives directly the mg. urea nitrogen per 100 c.c. blood. For high concentrations, the dilution factor $\left(\frac{25}{100}\right)$ must be corrected accordingly and the whole equation used in the calculation. (See colorimetric calculations p. 359.)

Alternative Titrimetric Procedure.—Instead of aerating the NH_3 into 15 c.c. of acidified water, the latter may be replaced by 10 c.c. of 0.01 N sulphuric acid. After the aeration is completed, titrate the excess acid with 0.01 N sodium or potassium hydroxide, using methyl red or alizarin as an indicator.[1] A blank determination, using water in place of blood, is run simultaneously. If 10 c.c. of standard acid are insufficient, repeat using 20 c.c.

Calculation.—Subtract the c.c. of 0.01 N NaOH used in titrating the unknown from the c.c. used in titrating the blank and multiplying by 7 to obtain the mg. of urea N per 100 c.c. of blood. If 20 c.c. of standard acid are used, subtract from 10 plus the blank, instead of as above.

3D. Determination of Blood Urea by Manometric Gas Analysis (Van Slyke). See p. 470.

4. **Determination of Preformed Creatinine.**—*Principle.*—A portion of the blood filtrate is treated with alkaline picrate solution and the color developed compared with that of a standard in a colorimeter.

Procedure.—Transfer 25 (or 50) c.c. of a saturated solution of purified picric acid[2] to a small, clean flask, add 5 (or 10) c.c. of 10 per cent sodium hydroxide, and mix. Transfer 10 c.c. of blood filtrate to a small flask or to a test tube, transfer 5 c.c. of the standard creatinine solution described below to another flask, and dilute the standard to 20 c.c. Then add 5 c.c. of the freshly prepared alkaline picrate solution to the blood filtrate, and 10 c.c. to the diluted creatinine solution. Let stand for 8 to 10 minutes and make the color comparison in the usual manner, never omitting first to ascertain that the two fields of the colorimeter are equal when both cups contain the standard creatinine picrate solution. The color comparison should be completed within 15 minutes from the time the alkaline picrate was added; it is, therefore, never advisable to work with more than three to five blood filtrates at a time.

When the amount of blood filtrate available for the creatinine determination is too small to permit repetition, it is of course advantageous or necessary to start with more than one standard. If a high creatinine should be encountered unexpectedly without several standards ready, the determination can be saved by

[1] The indicator may conveniently be incorporated in appropriate concentration in the preparation of the standard acid. 12 c.c. of 1 per cent alizarin solution per liter gives a satisfactory color.

[2] Picric acid may be purified as indicated in the section on Reagents and Solutions, Appendix.

diluting the unknown with an appropriate amount of the alkaline picrate solution—using for such dilution a picrate solution first diluted with two volumes of water—so as to preserve equality between the standard and the unknown in relation to the concentration of picric acid and sodium hydroxide.

One standard creatinine solution, suitable both for creatine and for creatinine determinations in blood, can be made as follows: Transfer to 500 c.c. volumetric flask 3 c.c. of the standard creatinine solution used for urine analysis (which contains 0.100 gm. of creatinine dissolved in 100 c.c. of 0.1N hydrochloric acid); add 50 c.c. of 0.1N hydrochloric acid, dilute to the mark with water, and mix. Transfer to a bottle and add four or five drops of toluene or xylene. Five c.c. of this solution contain 0.03 mg. of creatinine and this amount plus 15 c.c. of water represents the standard needed for the vast majority of human bloods, for it covers the range of 1 to 2 mg. per 100 c.c. In the case of unusual bloods representing retention of creatinine it is best to use smaller amounts of blood filtrate plus enough water to make the usual 10 c.c. correcting, the calculation given below, accordingly.

Calculation.—The reading of the standard in mm. multiplied by 1.5, and divided by the reading of the unknown, in mm., gives the amount of creatinine in mg. per 100 c.c. of blood. In connection with this calculation it is to be noted that the standard is made up to twice the volume of the unknown, so that each 5 c.c. of the standard creatinine solution, while containing 0.03 mg., corresponds to 0.015 mg. in the blood filtrate. If the unknown is set at 30, the reading of the standard divided by 20 gives the creatinine (in mg.) in 100 c.c. of blood.

Interpretation.—Normally creatinine is found in the blood to the extent of 1–2 mg. per 100 c.c. In early nephritis values of from 2–4 mg. are noted, and in severe nephritis 4–35 mg. Creatinine is more readily excreted by the kidneys than urea or uric acid, and an increase of creatinine to 4 or 5 mgs. or over per 100 c.c. of blood indicates a marked impairment of kidney function and a probable fatal termination within a relatively short time. Behre and Benedict[1] have shown that creatinine is not present in normal blood to anything like the extent indicated by the results of this procedure, the color obtained being chiefly due to other substances of unknown character. This does not affect the clinical value of the determination as an index of kidney impairment.

5. **Determination of Creatine Plus Creatinine.**—*Principle.*—The creatine of the blood filtrate is transformed to creatinine by heating with dilute HCl in an autoclave. The creatinine, preformed and from creatine, is then determined by treating with alkaline picrate as under preformed creatinine.

Procedure.—Transfer 5 c.c. of blood filtrate to a test tube graduated at 25 c.c. These test tubes are also used for urea and for sugar determinations. Add 1 c.c. of normal hydrochloric acid. Cover the mouth of the test tube with tin-foil and heat in the autoclave to 130°C. for 20 minutes or, as for the urea hydrolysis, to 155°C for 10 minutes. Cool. Add 5 c.c. of the alkaline picrate solution and let stand for 8 to 10 minutes, then dilute to 25 c.c. The standard solution is made by adding

[1]Behre and Benedict: *Jour. Biol. Chem.*, 52, 11, 1922.

to 10 c.c. of creatinine solution in a 50 c.c. volumetric flask, 2 c.c. of normal acid and 10 c.c. of the alkaline picrate solution and after 10 minutes, diluting to 50 c.c. The preparation of the standard must of course have been made first so that it is ready for use when the unknown is ready for the color comparison. The height of the standard, usually 20 mm., divided by the reading of the unknown and multiplied by 6 gives the "total creatinine" in mg. 100 c.c. blood.

In the case of uremic bloods containing large amounts of creatinine 1, 2, or 3 c.c. of blood filtrate, plus water enough to make approximately 5 c.c., are substituted for 5 c.c. of the undiluted filtrate.

Interpretation.—Total creatinine as determined by this method gives values of about 5–6 mg. per 100 c.c. for normal blood. High values for creatine have been obtained in severe nephritis.

6. Determination of Uric Acid (Benedict).[1] **Principle.**—A portion of the blood filtrate is treated directly with arseno-phosphotungstic acid reagent and sodium cyanide. The blue color obtained is compared with that given by a standard uric acid solution.

Procedure.—Five c.c. of blood filtrate are transferred to a test-tube of 18–20 mm. diameter and 5 c.c. of water added. Five c.c. of standard uric acid solution[2] containing 0.02 mg. of uric acid are also made up to 10 c.c. To both standard and unknown are added from a burette, 4 c.c. of 5 per cent sodium cyanide solution (poison!) containing 2 c.c. of concentrated ammonia per liter. To each tube is then added 1 c.c. of arsenic phosphoric acid tungstic acid reagent.[2] The contents of each tube should be mixed by one inversion immediately after addition of the reagent and placed at once in boiling water,[3] where the tubes should be left for 3 minutes after immersion of the last tube, but the time elapsing between the immersion of the first and last tubes should not exceed 1 minute. After the 3 to 4 minute heating, the tubes are removed and placed in a large

[1] Benedict: *Jour. Biol. Chem.*, 51, 187, 1922; 54, 233, 1922; 64, 215, 1925.

[2] Reagents for Uric Acid Determination.

Sodium Cyanide.—A 5 per cent solution containing 2 c.c. of concentrated ammonia per liter. Best prepared fresh every two months.

Uric Acid Reagent.—One hundred grams of pure sodium tungstate are placed in a liter flask and dissolved in about 600 c.c. of water. Fifty grams of pure arsenic pentoxide are now added, followed by 25 c.c. of 85 per cent phosphoric acid, and 20 c.c. of concentrated hydrochloric acid. The mixture is boiled for 20 minutes, cooled, and diluted to 1 liter. The reagent appears to keep indefinitely.

Uric Acid Standard Solutions.—The stock solution is made as follows: Dissolve 9 grams of pure crystallized disodium hydrogen phosphate and 1 gram crystallized sodium dihydrogen phosphate in 200–300 c.c. of hot water. Filter if not perfectly clear. Make up to about 500 c.c. with hot water and pour this hot or warm (but perfectly clear) solution upon exactly 200 mg. pure uric acid suspended in a few c.c. of water in a liter volumetric flask. Agitate a few minutes until uric acid completely dissolves. Cool, add *exactly* 1.4 c.c. of glacial acetic acid, dilute to mark and mix. Add about 5 c.c. of chloroform to prevent the growth of bacteria or moulds in the solution. Five c.c. of this solution contains exactly 1 mg. of uric acid. This solution should keep about 2 months unless in an excessively warm room.

The dilute standard containing 0.02 mg. uric acid in 5 c.c. is prepared as follows: 10 c.c. of the stock uric acid phosphate solution are measured into a 500 c.c. volumetric flask and the flask is about half filled with distilled water. Twenty-five c.c. of dilute HCl (1 volume of concentrated acid diluted to 10 volumes with water) are added, and the solution is diluted to 500 c.c. This solution should be prepared fresh every two weeks.

[3] Brown (*Jour. Biol. Chem.*, 68, 123, 1926) obtains lower values by allowing the solution to stand for 20 minutes at room temperature, instead of heating. Twice the quantity of filtrate is used.

beaker of cold water for 3 minutes and read in a colorimeter against the standard as soon as convenient (preferably within 5 minutes after removing from the cold water). It is best not to run more than 4 tubes at a time with one standard.

If clouding of the solutions should occur (usually the result of over-oxalating the blood) repeat the test adding another 5 c.c. of water to the standard as well as to the unknowns, just previous to heating. No change need be made in the calculation.

Calculation.—Employing the standard solution containing 0.02 mg. of uric acid and using 5 c.c. of the 1:10 blood filtrate the calculation is as follows: $\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 4 =$ mg. uric acid per 100 c.c. of blood. If the unknown is set at 20 mm. on the left, the reading of the standard is multiplied by 0.2 to give the required result.

Interpretation.[1]—Normal human blood usually contains from 2–3.5 mg. of uric acid per 100 c.c. In early interstitial nephritis values of from 3–10 mg. are noted. Uric acid may increase in the blood in this condition sooner than urea or creatinine, probably because it is less soluble and less readily excreted by the kidneys. The determination of uric acid is, therefore, of especial value in early nephritis. In severe nephritis values up to 25 mg. may be obtained.

In gout high uric acid values (4–10 mg.) are usually found. Determination of uric acid, therefore, is of value in the diagnosis of gouty arthritis prior to the stage of tophi formation. It must be borne in mind, however, that uric acid is similarly increased in early nephritis and that many cases of gout showing high uric acid values also show defective kidney function by other tests. The same difficulty is met with in considering the high values (2–8 mg.) obtained in other arthritic conditions, usually associated with increases in urea also. The existence of nephritis in such cases has not been entirely excluded and many typical cases of arthritis show values below 3 mg. Salicylates, and atophan (cincophen) tend to reduce the uric acid content of the blood.

The excessive breakdown of nuclear material in leukemia is accompanied by elevated uric acid values. In addition to the numerous conditions resulting in renal damage, such as mercury or lead poisoning, eclampsia, acute infections, malignancy, etc., the blood uric acid is also increased in certain cardiac conditions which ultimately involve the kidneys.

6A. Determination of Uric Acid. Folin's Direct Method.[2] **Principle.**—The principle is the same as that of the preceding method *i.e.* the reduction of a

[1] See reviews by Folin, Berglund and Derick: *Jour. Biol. Chem.*, 60, 361, 1924, and Rose' *Physiol. Rev.*, 3, 544, 1923

[2] Folin: *Jour. Biol. Chem.*, 54, 153, 1922. Folin: Laboratory Manual of Biological Chemistry, New York, 1925.

phosphotungstic acid solution by uric acid giving a blue compound which is estimated colorimetrically. Estimations directly on blood filtrates have been criticized as giving too high values due to a sulphur containing substance present which gives the same reaction.[1] The procedure is unreliable for animal bloods and gives somewhat high results for human blood but is much more convenient than the isolation method (see next method). By removing traces of molybdenum from the reagent the specificity is increased.[2]

Procedure.—Fill a 500–1000 c.c. beaker two thirds full of water and heat to boiling. Fill a burette with 15 per cent sodium cyanide solution and another with uric acid reagent. Transfer 5 c.c. of blood filtrate to a test tube graduated at 25 c.c. and 5 c.c. of standard uric acid (0.02 mg. uric acid) to a similar tube. To each tube add 2 c.c. of cyanide solution and 2 c.c. of water. Mix well as cyanide goes to the bottom. Add 1 c.c. of uric acid reagent[3] to each tube dropping it from

[1] Benedict, Newton and Behre: *Jour. Biol. Chem.*, 67, 267, 1926. Hunter and Eagles: *Jour. Biol. Chem.*, 65, 623, 1925. Bulmer, Eagles and Hunter: *Jour. Biol. Chem.*, 63, 17, 1925. Benedict: *Jour. Biol. Chem.*, 64, 215, 1925; Hunter and Eagles (Personal communication) have confirmed the presence of sulphur in the interfering substance.

[2] Folin and Trimble: *Jour. Biol. Chem.*, 60, 473, 1924.

[3] Reagents for Uric Acid Determination.

1. 15 *per cent Sodium Cyanide Solution in* 0.1 *N Sodium Hydroxide.* At least 1–2 days but not more than a month old. Use C.P. cyanide. Merck's is good.

2. *Standard Uric Acid Solution* (5 c.c. = 0.02 mg.). Half fill a 250 c.c. volumetric flask with distilled water. With accurate Ostwald pipette add 1 c.c. of standard stock solution of uric acid (see below). Then add 10 c.c. $\frac{2}{3}$ N sulphuric acid, dilute to volume and mix. This will keep at least a week. With not more than 1 c.c. of formalin added in the preparation the solution will keep almost as well as the stock solution.

3. *Stock Solution of Uric Acid.*—Transfer exactly 1 gm. of uric acid to a dry funnel inserted into a volumetric flask (1000 c.c.) and shake uric acid into flask as well as possible. Dissolve 0.6 gm. lithium carbonate in about 120 c.c. hot water and filter. Dilute with about 60 c.c. water and heat to 65°. Warm volumetric flask in hot water and add hot lithium carbonate solution rinsing funnel and watch glass used in weighing free from last traces of uric acid. Shake until dissolved (usually 5–15 minutes). Cool and dilute to about 800 c.c. Add 10 c.c. clear 37–40 per cent formaldehyde (Merck) and mix. Dilute 15 c.c. concentrated sulphuric acid with about 100 c.c. of water and cool. Add to urate formalin mixture, dilute to volume and mix. The solution will keep at least for several months.

4. *Uric Acid Reagent.*—For ordinary clinical determinations use reagent as described on p. 737. For highest accuracy use the following. Transfer 100 gm. normal sodium tungstate [$Na_2WO_4\ 2H_2O$] and 160 c.c. of water to a 500 c.c. flask. Add 50 c.c. of 85 per cent phosphoric acid, a few c.c. at a time with cooling under running water to prevent appreciable rise in temperature. Connect flask with gas wash bottle (half filled with water), to a Kipp hydrogen sulphide generator. The flask has a two hole stopper and has an outlet tube which can be opened or closed with a pinch cock. The inlet tube goes to bottom of flask. The stoppers must be gas tight. Open stopcock of generator and then open pinchcock momentarily and close again, allowing hydrogen sulphide to bubble through the solution. Repeat opening and closing of pinchcock 6–7 times to remove all air from the flask and fill it with hydrogen sulphide. Then close pinchcock but leave stopcock of generator open. Then hydrogen sulphide will be produced by the generator only as fast as it is absorbed by the solution. Leave over night. Solution takes on deep blue color. The next morning disconnect the apparatus and filter the solution into a 500 c.c. flask. Avoid transferring the precipitate to the filter paper until most of the liquid has passed through. Allow the precipitate to drain completely, but do not wash it. Insert a 10 cm. funnel and in this funnel place a 200 c.c. flask, filled with cold water and boil the contents of the flask gently, but continuously, over a microburner for 1 hour. Funnel and flask serve as a condenser. Boiling must not greatly reduce the volume of solution. Filter the hot solution from the additional small quantity of molybdenum sulphide which comes out during the boiling. This time washing is permissible. Wash with small quantities of water until most of the blue color has been removed from the filter. To remove blue color from filtrate heat to boiling, remove flame, and decolorize by adding bromine and shaking vigorously. Add only 2–3 drops at a time. When enough bromine has been added a clear light yellow solution is obtained. Boil gently for ten minutes to remove excess bromine and cool.

Transfer to a liter beaker 25 gm. lithium carbonate. Add 50 c.c. of 85 per cent phosphoric acid and then 200 c.c. of water. Boil off the carbon dioxide and cool. Mix the two

the burette directly into the solution in the tube (not down the sides). Let stand for 2 minutes at room temperature, then put in boiling water bath for 2 minutes (not longer). Cool at once and dilute promptly to the 25 c.c. mark. Mix. Compare in a colorimeter.

Calculation.

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 4 = \text{mg. uric acid in 100 c.c. of blood.}$$

When the unknown is set at 20 mm., the calculation becomes :—0.2 multiplied by the reading of the standard. If the standard reading is greater than 40, repeat using 3 c.c. of filtrate and 2 c.c. of water. If less than 10 use 10 c.c. of filtrate and 5 c.c. of standard with 5 c.c. of water.

6B. Determination of Uric Acid (Folin's Isolation Procedure).[1]—*Principle.*—Uric acid is precipitated as silver urate, directly from the blood filtrate. The uric acid is set free by means of acid chloride solution and determined colorimetrically after addition of phosphotungstic acid which gives a blue solution. Since the uric acid is isolated from the interfering substances referred to in the preceding method, this procedure is more accurate.

Procedure.—Transfer 5 c.c. of the blood filtrate to a centrifuge tube. With a blood pipette or a small cylinder add 7 c.c. of the silver lactate solution.[2] No stirring is necessary or desirable. Let settle for 1 to 2 minutes and then centrifuge. All the uric acid, down to the last trace will now be in the precipitate.

Decant the supernatant solution as completely as possible and add 1 c.c. of a 10 per cent solution of sodium chloride in N/10 hydrochloric acid. Stir *thoroughly* with a fine glass rod; add 4 c.c. of water, and stir again. Centrifuge.

Pour the supernatant solution as completely as possible into a test tube graduated at the 25 c.c. mark. Transfer 5 c.c. of the standard uric acid solution to another similar test tube. Proceed as described in the direct method, (6A).

Calculation.—$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 4 = \text{mg. uric acid in 100 c.c.}$ of blood.

7. Determination of Amino Acids.[3]—**Principle.**—The color developed by amino acids in the presence of β-naphthaquinone sulphonic acid and alkali is made the basis of this method.

solutions and dilute to 1 liter. The reagent should give but a very faint bluish tint with 0.2 mg. resorcinol. For determination of uric acid in silver precipitates from human urine or for clinical determinations on human blood the removal of molybdenum may not be essential as uric acid gives a much stronger color than phenols. However if a large trace of molybdenum is present errors up to 1 mg. per 100 c.c. of blood may occur.

[1] Folin: *Jour. Biol. Chem.*, 54, 153, 1922. For a comparative study of the *direct* and *isolation* procedures see Wiener and Wiener: *J. Lab. Clin. Med.*, 11, 1035, 1926.

[2] A solution of silver lactate is prepared as follows:

Dissolve 100 g. of silver lactate in about 700 c.c. of warm water. To 100 c.c. of 85 per cent lactic acid add 100 c.c. of 10 per cent sodium hydroxide. Pour this partly neutralized lactic acid into the silver lactate solution, dilute to one liter and set aside to allow the sediment, always present, to settle. Use only the clear supernatant solution. Also required is a solution of 10 per cent sodium chloride in N/10 hydrochloric acid. Other reagents used are the same as for Folin's direct method.

[3] Folin: *Jour. Biol. Chem.*, 51, 377, 1922.

Procedure.—Into a large test-tube, graduated at 25 c.c., introduce 10 c.c. of blood filtrate. To another tube transfer 1 c.c. of standard amino-acid solution[1] and 8 c.c. of water. Add 1 drop of phenolphthalein solution to each. Add 1 c.c. of special 1 per cent sodium carbonate solution to the standard, and to the blood filtrate add the carbonate solution drop by drop, until the same pink color as in the standard is obtained (6–8 drops are usually required). Add 2c. c. of freshly prepared 0.5 per cent β-naphthaquinone solution to each, mix, and set aside over night in a perfectly dark place. The next day add first 2 c.c. of acetic acid-acetate solution and then 2 c.c. of 4 per cent thiosulphate solution to each tube. This decolorizes excess of reagent. Dilute to 25 c.c. mark, mix, and make color comparison.

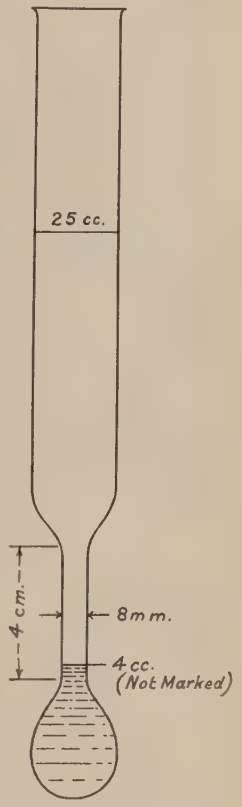


FIG. 119.—FOLIN-WU SUGAR TUBE.

(Folin and Wu: *Jour. Biol. Chem.*, 41, 367, 1920).

Calculation.

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 7 = \text{mg. amino-acid nitrogen in 100 c.c. of blood.}$$

Interpretation.—Blood normally contains from 5–8 mg. amino-acid nitrogen per 100 c.c. A slight increase is noted after ingestion of protein foods, and high values have been noted in uremic nephritis,[2] although in a study of an extended series of cases Greene, Sandiford and Ross[3] have found a remarkable constancy in amino N even in pathological conditions. Increases were noted in leucemia and in acute yellow atrophy of the liver, due to tissue autolysis.

8. Determination of Glucose (Folin and Wu).[4]

Principle.—The protein-free blood filtrate is heated with alkaline copper solution, using a special tube to prevent reoxidation. The cuprous oxide formed is treated with a molybdate phosphate solution, a blue color being obtained which is compared with that of a standard.

[1] Reagents used in amino-acid determination.

(*a*) *Standard Amino-acid Solution.*—A solution containing 0.07 mg. nitrogen per c.c. Glycine may be used or leucine, phenylalanine or tyrosine. Glycine of doubtful purity may be recrystallized by dissolving in water and adding 0.5-1 volume of alcohol. The amino acid standard is made up in 0.1 N HCl containing 0.2 per cent sodium benzoate.

(*b*) *Special Sodium Carbonate Solution.*—Fifty c.c. of approximately saturated solution are diluted to 500 c.c. Titrate 20 c.c. of 0.1 N hydrochloric acid with the carbonate, using methyl red as an indicator. Dilute so that 8.5 c.c. are equivalent to 20 c.c. of 0.1 N acid. The carbonate solution is about 1 per cent.

(*c*) *Amino-acid Reagent.*—A 0.5 per cent solution of the sodium salt of β-naphthaquinone sulphonic acid freshly prepared. The quinone itself may be prepared according to a method described in the original paper.

(*d*) *Special Acetic Acid-Acetate Solution.*—Dilute 100 c.c. of 50 per cent acetic acid with an equal volume of 5 per cent sodium acetate solution.

(*e*) *Sodium Thiosulphate Solution.*—A 4 per cent solution of the crystalline salt.

[2] Bock, *Jour. Biol. Chem.*, 29, 191, 1917.

[3] Greene, Sandiford and Ross, *Jour. Biol. Chem:* 58, 845, 1924.

[4] Folin and Wu: *Jour. Biol. Chem.*, 41, 367, 1920. See Folin's modification of this method, 8B (p. 384). For an adaptation of the Shaffer-Hartmann sugar method (see p. 749) to blood see Haskins and Holbrook: *J. Lab. Clin. Med.*, 8, 747, 1923 and Bugbee and Simond: *ibid.*, 11, 990, 1926.

Procedure.—Transfer 2 c.c. of the tungstic acid blood filtrate to a blood sugar test tube of the type illustrated in Fig. 119 and to two other similar test tubes (graduated at 25 c.c.) add 2 c.c. of standard sugar solutions containing respectively 0.2 and 0.4 mg. of glucose.[1] To each tube add 2 c.c. of the alkaline copper solution.[2]

The surface of the mixtures must now have reached the constricted part of the tube. If the bulb of the tube is too large for the volume (4 c.c.) a little, but not more than 0.5 c.c. of a diluted (1:1) alkaline copper solution may be added. If this does not suffice to bring the contents to the narrow part, the tube should be discarded. Test tubes having so small a capacity that 4 c.c. fills them above the neck should also be discarded. Transfer the tubes to a boiling water bath and heat for 6 to 8 minutes. Add immediately (before cooling) to each test tube 2 c.c. of phosphomolybdic acid solution.[3] When the cuprous oxide is dissolved cool and dilute the resulting blue solutions to the 25 c.c. mark, insert a rubber stopper, and mix. It is essential that adequate attention be given to this mixing because the greater part of the blue color is formed in the bulb of the tube. Compare in a colorimeter using the standard which most nearly matches the unknown.

The two standards given representing 0.2 and 0.4 mg. of glucose are adequate for practically all cases. They cover the range from about 70 to nearly 400 mg. of glucose per 100 c.c. of blood.

Calculation.—$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 100 =$ milligrams of glucose per 100 c.c. of blood, if the weaker standard (0.2 mg.) is used. For the stronger standard, multiply the ratio of colorimetric readings by 200 instead of 100.

These calculations are not strictly accurate because the color produced does not vary directly with the concentration of glucose. Oser and Karr[4] have suggested correcting the values obtained in this method by reference to curves con-

[1] *Standard Sugar Solutions.*—Three standard sugar solutions should be on hand: (1) a stock solution, 1 per cent glucose or invert sugar, made up in 0.25 per cent benzoic acid solution; (2) a solution containing 0.2 mg. of sugar in 2 c.c. (5 c.c. of the stock solution diluted to 500 c.c. with 0.25 per cent benzoic acid solution); (3) a solution containing 0.4 mg. of sugar in 2 c.c. (5 c.c. of the stock solution diluted to 250 c.c.). The invert sugar solution has the advantage that it can be easily prepared from cane sugar, which is pure. When good quality glucose is available, it is, of course, the one to use. Merck's *highest purity* dextrose is satisfactory.

[2] *Alkaline Copper Solution.*—Dissolve 40 gm. of pure anhydrous sodium carbonate in about 400 c.c. of water and transfer to a liter flask. Add 7.5 gm. of tartaric acid, and when the latter has dissolved add 4.5 gm. of crystallized copper sulphate. Mix and make up to a volume of 1 liter. If the chemicals used are not pure a sediment of cuprous oxide may form in the course of 1 or 2 weeks. If this should happen, remove the clear supernatant reagent with a siphon, or filter through a good quality filter paper. The reagent seems to keep indefinitely. To test for the absence of cuprous copper in the solution, transfer 2 c.c. to a test tube and add 2 c.c. of the molybdate phosphate solution; the deep blue color of the copper should almost completely vanish. In order to forestall improper use of this reagent attention should be called to the fact that it contains extremely little alkali, 2 c.c. by titration (using the fading of the blue copper tartrate color as indicator), requiring only about 1.4 c.c. of normal acid.

[3] *Phosphomolybdic Acid Solution.*—To 35 gm. molybdic acid and 5 gm. sodium tungstate, add 200 c.c. of 10 per cent sodium hydroxide and 200 c.c. of water. Boil vigorously for 20 to 40 minutes so as to remove nearly the whole of the ammonia present in the molybdic acid. Cool, dilute to about 350 c.c., and add 125 c.c. of concentrated (85 per cent) phosphoric acid. Dilute to 500 c.c.

[4] Oser and Karr: *Jour. Biol. Chem.*, 67, 319, 1926.

structed from readings of glucose solutions of known concentration. Tables from which these curves may be constructed are given in their paper.

Folin claims that the modified molybdate solution (see p. 384) gives a more dependable proportionality between color intensity and concentrations.

Interpretation.[1]—Depending on the method used, the glucose concentration of normal blood varies over a wide range (70 to 120 mg. per 100 c.c.), but for any particular method the normal range covers about 30 mg. That is, for the Folin-Wu method 90 to 120 mg. may be regarded as the normal limits; for Folin's modification of this method, 80 to 110; for Benedict's copper reduction method, 70 to 100. The lower values are probably the better ones, inasmuch as the methods have been revised to make the conditions more specific for reduction by glucose. In mild diabetes values of from 140 to 300 mg. are obtained, and in severe diabetes values up to 1200 mg. Hyperglycemia is also observed in severe nephritis, hyperthyroidism, pancreatic disease and in certain hepatic disorders. Hypoendocrine disturbances (excluding the pancreas) may result in low levels of blood glucose. Among these conditions are Addison's disease, hypopituitarism, cretinism, myxedema and muscular dystrophy.

The normal threshold of blood glucose is about 160 to 180 mg. per 100 c.c. Above this level glucose "spills over" into the urine. The threshold is frequently raised in diabetes, so that in these cases it is inadvisable to place too much reliance on urinalyses alone. "Renal diabetes" or "renal glycosuria" is a condition in which the threshold is lowered, glycosuria occurring with little or no hyperglycemia.

The mechanism by which insulin exerts its action in reducing the blood glucose is still undetermined. The process effects not only the carbohydrate mobilization, but also the oxidation of fats, causing a reduction of lipemia and acetone body formation, and a consequent rise in the lowered alkali reserve.[2]

For carbohydrate tolerance tests, see p. 388.

8A. Benedict[3] Modification of the Folin and Wu Blood Sugar Method. Principle.—A new copper reagent is used which is less readily reduced by substances in the blood filtrates other than glucose, and hence is believed to give values representing more closely the true glucose concentration. By this method the normal blood sugar averages 75 mg. per 100 c.c. Benedict believes that even this may be too high, and that the correct value is 60 mg. or less.[4]

[1] For a review, see Macleod *Physiol. Rev.* 1, 208, 1921.

[2] For an important contribution to the literature on this subject, see the "insulin number" of the *Journal of Metabolic Research*, 2, 547–985, 1922, which appeared in May, 1923.

[3] Benedict: *Jour. Biol. Chem.*, 68, 759, 1926. Harned: *Jour. Biol. Chem.*, 65, 555, 1925, precipitates the blood proteins with mercuric nitrate and obtains sugar values similar to those of Benedict.

[4] Wagner, *J. Metab. Res.*, 5, 253, 1924 (appeared July, 1926), by a fermentation method, obtains plasma sugar values about 10 per cent lower than Benedict's.

Procedure.—Two c.c. of the 1:10 tungstic acid filtrate are measured into a Folin-Wu sugar tube followed by 2 c.c. of the copper reagent.[1] Mix by side to side shaking for a moment and put tubes in boiling water for 5 minutes. The reduced copper is held in solution by the ammonium salt. Cool by immersion in cold water and add 2 c.c. of the complex tungstic acid reagent[2] whereupon the color development is practically immediate. After 1 to 2 minutes, dilute to the 25 c.c. mark mix thoroughly and compare in a colorimeter with a standard solution treated in the same way. The Folin and Wu standards (see p. 382) may be employed.[3]

Calculation.—The calculation as given for the Folin-Wu method is employed.

Interpretation.—See above. Normal values for blood glucose by this procedure range between 60 and 90 mg. per 100 c.c.

8B. Folin[4] Modification of the Folin and Wu Blood Sugar Method. Principle. By the use of more delicately adjusted reagents, the Folin-Wu method has been modified to give normal values for blood glucose which are lower and presumably more accurate.

Procedure.—Blood filtrates must be nearly neutral, i.e., 10 c.c. should require about 0.2 c.c. of 0.1 N NaOH for neutralization.. If not already neutral, filtrates may be conveniently made so by the addition of a drop of phenolphthalein to 2 c.c. of filtrate, followed by the number of drops of 0.1 N NaOH required to give the pink endpoint. Then add the same number of drops of alkali to the sugar tubes before introducing the filtrates. Under constant laboratory conditions the latter procedure may be followed for all filtrates without preliminary titration. Transfer 2 c.c. of nearly neutral or neutralized blood filtrate to a Folin-Wu sugar tube, and to another add 2 c.c. of standard glucose solution.[5] Add 2 c.c. of the Folin copper solution[6] to each tube and heat in a boiling water bath for 10 minutes. Cool and add 2 c.c. of acid molybdate reagent[7] to each tube. As soon as the visi-

[1] *Alkaline Copper Reagent.*—Dissolve 200 gm. sodium citrate and 60 gm. anhydrous sodium carbonate in about 800 c.c. of water. Dissolve 6.5 gm. pure crystallized copper sulphate separately in about 100 c.c. of water and add to the other solution while stirring. Add 9.0 gm. ammonium chloride, dilute the solution to 1 liter and mix thoroughly. Not more than 1 month before use, add to a small reagent bottle containing about 100 c.c. of this reagent, about 2.5–3.0 gm. sodium sulphite (measured with a porcelain spoon known to hold about that amount). The reagent is now ready for use. For occasional sugar determinations, omit addition of sulphite to the reagent, but introduce 5 drops of 20 per cent sodium sulphite into each sugar tube prior to adding the other solutions.

[2] *Complex Tungstic Acid Reagent.*—In a liter flask dissolve 100 gm. pure sodium tungstate in about 600 c.c. of water. Add 50 gm. pure arsenic pentoxide, 25 c.c. of 85 per cent phosphoric acid and 20 c.c. of concentrated hydrochloric acid. Boil mixture 20 minutes. Cool, add 60 c c. commercial formalin, 45 c.c. concentrated hydrochloric acid and 40 gm. sodium chloride. Dissolve, dilute to 1 liter and mix. This reagent contains more formalin and has a higher acidity and specific gravity than the Benedict uric acid reagent.

[3] Benedict uses pure aqueous (instead of benzoic acid) solutions of glucose as standards, preserving with toluene.

[4] Folin: *Jour. Biol. Chem.*, 67, 357, 1926.

[5] The stock 1.0 per cent glucose solution may be made up in saturated benzoic acid, but the dilute standards must be made with water, adding a few drops of formalin or toluene as a preservative. This avoids the complicating factor of acidity.

[6] *Alkaline Copper Tartrate Solution.*—Dissolve 12 gm. of Merck's sodium tartrate (or 15 gm. of Rochelle salt) together with 7 gm. of anhydrous sodium carbonate and 20 gm. of sodium bicarbonate in 600 to 700 c.c. of distilled water. Transfer this solution to a volumetric liter flask, and to it add 5 gm. of copper sulphate previously dissolved in about 200 c.c. of water. Stopper the flask and shake for a few moments. Dilute to volume and mix.

[7] *Acid Molybdate Reagent.*—Dissolve 150 gm. of sodium molybdate $Na_2MoO_42H_2O$, in 300 c.c. of distilled water. Filter through a 15 cm. quantitative filter paper into a flask (capacity 1 liter) and wash with 75 c.c. of water. To the sodium molybdate solution in the flask add 2 or 3 drops of bromine and shake for a few minutes till the bromine has dissolved. Let stand for an hour to complete the oxidations produced by the hypobromite. Then add,

ble evolution of CO_2 has ceased (about 1 minute), dilute to the 25 c.c. mark, mix and make the color comparison.

Calculation.—The calculation as given for the Folin-Wu method is used. The new acid molybdate solution is said to give a more dependable proportionality of color for different amounts of cuprous oxide.

Interpretation.—See above. Normal values for blood glucose by this method range between 70 and 100 mg. per 100 c.c.

8C. Determination of Glucose in Finger-tip Blood.[1] **Principle.**—The Folin-Wu method is adapted to one-fourth the quantities of material ordinarily used.

Procedure.—0.1 c.c. of blood from a finger or ear prick is drawn into a micropipette calibrated at 0.1 and 0.8 c.c., followed by distilled water to the 0.8 c.c. mark. The hemolyzed blood is ejected into a centrifuge tube and 0.1 c.c. of 10 per cent sodium tungstate and the same volume of 2/3 N sulphuric acid are added as in the usual Folin-Wu precipitation. The tube is centrifuged, and the determination conducted on 0.5 c.c. of the supernatant fluid and on 0.5 c.c. of the standard glucose solutions, in tubes graduated at 6.25 c.c. and constricted at 1.0 c.c. The Folin-Wu technique is followed, using 0.5 c.c. of copper reagent and phosphomolybdic acid reagent.

Calculation.—The calculation is the same as in the Folin-Wu method, inasmuch as the supernatant fluid represents a 1 : 10 dilution of blood.

Interpretation.—See p. 383. It should be noted that finger-tip blood resembles venous blood only in the fasting state. During absorption or in a glucose tolerance test, finger-blood is arterial in character and shows higher glucose values than the venous blood leaving the muscle tissue. It is believed that in the performance of their glycogenetic function, muscle cells remove glucose from the blood.[2]

8D. Determination of Sugar. Method of Hagedorn and Jensen.[3] **Principle.**—The blood protein is precipitated with zinc hydroxide. The filtrate is heated with potassium ferricyanide solution and the amount of ferricyanide reduced is determined by adding an iodide solution and titrating the iodine set free with sodium thiosulphate. The principal reaction is $2H_3Fe(CN)_6 + 2HI = 2H_4Fe(CN)_6 + I_2$. The reversal of the reduction reaction is prevented by precipitation of the ferrocyanide formed as a zinc salt. Folin-Wu blood filtrates may also be used.[4]

Procedure.—Into a test tube (15 × 150 mm.) pipette 1 c.c. of N/10 NaOH and 5 c.c. 0.45 per cent zinc sulphate solution. A gelatinous precipitate of zinc hydroxide forms. 0.1 c.c. of blood from a capillary pipette[5] is introduced, the pip-

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with shaking, 225 c.c. of 85 per cent phosphoric acid. The surplus bromine is set free and imparts a yellow color to the solution. After all the phosphoric acid has been added, add also 150 c.c. of cooled 25 per cent sulphuric acid (one volume of concentrated sulphuric acid added to three volumes of water). Remove the surplus bromine by aeration for about half an hour. Finally, add 75 c.c. of 99 per cent acetic acid, mix, and dilute to a volume of 1 liter.

[1] Byrd: *Jour. Am. Med. Assn.*, 83, 508, 1924. The necessary apparatus for this method may be obtained from the Central Scientific Co., Chicago, Ill.

[2] Foster: *Jour. Biol. Chem.*, 55, 291, 303, 1923.

[3] Hagedorn and Jensen: *Biochem. Zeit.*, 135, 46, 1923 and 137, 92, 1923.

[4] Hiller, Linder and Van Slyke: *J. Biol. Chem.*, 64, 625, 1925.

[5] The length of the 0.1 c.c. pipette from the tip to the mark should be about 10–12 cm. and over all length about 20 cm. Calibrate as follows: With the pipette measure out 0.1 c.c. of N/10 potassium iodate solution into 10 c.c. of water. Add acid and KI solution in the usual way and titrate with N/200 thiosulphate. Some of the same iodate solution is then diluted to N/200 and exactly 2.0 c.c. of this in 10 c.c. of water is titrated with the same

C.c. 0.005 N $Na_2S_2O_3$ USED AND MG. GLUCOSE PRESENT

c.c.	0.00	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09
0.0	0.385	0.382	0.379	0.376	0.373	0.370	0.367	0.364	0.361	0.358
0.1	0.355	0.352	0.350	0.348	0.345	0.343	0.341	0.338	0.336	0.333
0.2	0.331	0.329	0.327	0.325	0.323	0.321	0.318	0.316	0.314	0.312
0.3	0.310	0.308	0.306	0.304	0.302	0.300	0.298	0.296	0.294	0.292
0.4	0.290	0.288	0.286	0.284	0.282	0.280	0.278	0.276	0.274	0.272
0.5	0.270	0.268	0.266	0.264	0.262	0.260	0.259	0.257	0.255	0.253
0.6	0.251	0.249	0.247	0.245	0.243	0.241	0.240	0.238	0.236	0.234
0.7	0.232	0.230	0.228	0.226	0.224	0.222	0.221	0.219	0.217	0.215
0.8	0.213	0.211	0.209	0.208	0.206	0.204	0.202	0.200	0.199	0.197
0.9	0.195	0.193	0.191	0.190	0.188	0 186	0.184	0.182	0.181	0.179
1.0	0.177	0.175	0.173	0.172	0.170	0.168	0.166	0.164	0.163	0.161
1.1	0.159	0.157	0.155	0.154	0.152	0.150	0.148	0.146	0.145	0.143
1.2	0.141	0.139	0.138	0.136	0.134	0.132	0.131	0.129	0.127	0.125
1.3	0.124	0.122	0.120	0.119	0.117	0.115	0.113	0.111	0.110	0.108
1.4	0.106	0.104	0.102	0.101	0.099	0.097	0.095	0.093	0.092	0.090
1.5	0.088	0.086	0.084	0.083	0.081	0.079	0.077	0.075	0.074	0.072
1.6	0.070	0.068	0.066	0.065	0.063	0.061	0.059	0.057	0.056	0.054
1.7	0.052	0.050	0.048	0.047	0.045	0.043	0.041	0.039	0.038	0.036
1.8	0.034	0.032	0.031	0.029	0.027	0.025	0.024	0.022	0.020	0.019
1.9	0.017	0.015	0.014	0.012	0.010	0.008	0.007	0.005	0.003	0.002

ette being washed out twice with the mixture and blown empty. Put in a boiling water bath for 3 minutes. Filter on a funnel of 3-4 cm. diameter, prepared with a small filter of washed, moistened, not tightly pressed cotton, into a test tube (30 × 90 mm.). Wash funnel and filter with two 3 c.c. portions of water. Add 2 c.c.

thiosulphate as before. The two titrations should agree within experimental error (about 0.5 per cent) if the pipette is accurate.

Solutions Required. *For Protein Precipitation.—N/10 NaOH. Zinc sulphate* 0.45 per cent. These solutions are best prepared every eight days by dilution of 2 N NaOH and a zinc sulphate solution containing 45 grams of the salt in 100 c.c. of solution. *For Sugar Determination. Potassium ferricyanide* 1.65 gm. and sodium carbonate (fused) 10.6 gm. in 1000 c.c. of water. Protect from light. *Iodide-sulphate-chloride solution:* KI, 5.0 gm. zinc sulphate, 10.0 gm. NaCl 50.0 gm. Water to make 200 c.c. It is best to prepare the solution without iodide and add the latter to portions of the solution as required. Free iodine can be almost completely removed by filtering through thick paper. The blank will take care of smaller errors. *Acetic acid solution:* 3 c.c. of acetic acid (iron free) with water to make 100 c.c. *Starch solution:* 1 gm. of soluble starch dissolved in 100 c.c. of saturated NaCl solution. *Sodium thiosulphate solution:* 9.7 gm. sodium thiosulphate in 500 c.c. water. 0.005 *N Potassium iodate:* This solution is permanent and is used to check the thiosulphate and ferricyanide solutions which keep less well. It alone need therefore be prepared very accurately. 0.3566 gm. potassium iodate (water-free) is dissolved in water to make 2000 c.c.

Chemicals of highest purity must be used. Sodium carbonate is best recrystallized and fused in platinum. Acetic acid should be tested for iron as should also the zinc sulphate, sodium chloride, and potassium iodide. The iodide should also be tested for iodate. Ordinary quantities of the mixed solutions will then give no test for iodine with starch but will give a test if 0.01 c.c. of ferricyanide is added. Potassium ferrocyanide is prepared by washing crystals of the ordinary product with water, dissolving in water with heat and filtering the boiling solution through paper, previously carefully washed with boiling water, into an evaporating dish set in ice cold water. The fine crystals are filtered off with suction on another washed paper and again recrystallized. Dry at 50°. Keep away from sunlight during course of preparation.

alkaline potassium ferricyanide solution and heat in a boiling water bath for 15 minutes. Cool and add 3 c.c. of the iodide-sulphate solution and 2 c.c. of 3 per cent acetic acid solution. Titrate with 0.005 N sodium thiosulphate using as an indicator 2 drops of one per cent solution of soluble starch in saturated sodium chloride solution.

Calculation.—Determine the blank obtained by carrying through the whole determination but without the addition of blood.[1] Multiply A (thiosulphate burette reading) by factor for the thiosulphate (2.00/c.c. thiosulphate required for 2 c.c. of 0.005 N iodate). Express this value (B) for unknown and for blank as mg. glucose by consulting the table which follows. Subtract glucose value of blank from glucose value of unknown. The difference is mg. glucose in 0.1 c.c. of blood. Or calculate as follows: $2.00 - B = x$ (c.c. 0.005 N ferricyanide reduced). 2.00 c.c. of ferricyanide is reduced by 0.385 mg. glucose. Therefore

$$\text{mg. glucose} = 0.385 \times \frac{x}{2.0} \text{ per cent.}$$

8E. Picric Acid Method (Benedict[2]).—Principle.—The red color obtained by heating a glucose solution with picric acid and sodium carbonate is employed as the basis of the colorimetric determination. The blood protein is removed by precipitation with picric acid. The sugar values obtained by this method are doubtless too high.

Procedure.—Two c.c. of blood are aspirated through a hypodermic needle[3] and a piece of rubber tubing into an Ostwald pipette, a little powdered potassium oxalate in the tip of the pipette preventing clotting. The blood is drawn up a little above the mark and the end of the pipette is closed with the finger. After the rubber tubing and needle are disconnected, the blood is allowed to flow back to the mark and is discharged at once into a 25 c.c. volumetric flask, or into a large test-tube graduated at 12.5 c.c. and at 25 c.c. The pipette is twice rinsed with distilled water, these washings being added to the blood. The contents of the flask are shaken to insure thorough mixing and a consequent laking or hemolysis of the blood, which is practically complete after a minute or two. A solution of sodium picrate and picric acid[4] is added to the 25 c.c. mark (using a few drops of alcohol to dispel foam if necessary) and the mixture thoroughly shaken. After a minute or two (or longer) the mixture is poured upon a dry filter, and the clear filtrate collected in a dry beaker. Exactly 8 c.c. of the filtrate are measured into a large test-tube bearing graduations at the 12.5 c.c. and 25 c.c. marks, and 1 c.c. of 20 per cent (anhydrous) sodium carbonate solution is added. The tube is plugged with cotton and immersed in boiling water for 10 minutes.[5]

[1] Acetone and β-hydroxybutyric acid do not reduce the reagent. One mg. of uric acid gives a reduction equal to that of 0.53 mg. glucose, and one mg. creatinine a reduction equal to 0.47 mg. glucose.

[2] Benedict: *Jour. Biol. Chem.*, 34, 203, 1918. Lewis and Benedict: *Jour. Biol. Chem.*, 20, 61, 1915. For other modifications see Pearce: *Jour. Biol. Chem.*, 22, 525, 1915, and Myers and Bailey: *Jour. Biol. Chem.*, 24, 147, 1916.

[3] It may be more convenient to draw about 5 c.c. of blood directly into a test-tube containing a little finely powdered potassium oxalate and removing 2 c.c. portions of this with the Ostwald pipette.

[4] To prepare the picrate-picric acid solution, place 36 gm. of dry powdered picric acid in a liter flask or stoppered cylinder, add 500 c.c. of 1 per cent sodium hydroxide solution and 400 c.c. of hot water. Shake occasionally until dissolved. Cool and dilute to 1 liter.

[5] Longer heating up to half an hour makes no change in the color.

It is then removed, and the contents are cooled under running water and diluted to 12.5 c.c. or to 25 c.c. depending on the depth of color.[1] At any time within a half hour the colored solution is compared in a colorimeter with a suitable standard solution.

The standard solution may be simultaneously prepared from pure glucose by treating 0.64 mg. of glucose in 4 c.c. of water with 4 c.c. of the picrate-picric acid solution and 1 c.c. of the carbonate, and heating for 10 minutes in boiling water and then diluting to 12.5 c.c. A permanent standard solution may be prepared from picramic acid or from potassium dichromate.[2] The potassium dichromate standard does not match the unknown with absolute exactness, but can be employed with satisfactory results when pure picramic acid is not obtainable.

Calculation.—If directions are followed exactly the calculation is as follows:

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 100 = \text{mg. glucose per 100 c.c. blood.}$$

Set the unknown at 20 on the left and 5 times the reading of the standard equals the glucose concentration in mg. per 100 c.c. Where the final dilution of the unknown is made to 25 c.c. instead of 12.5 c.c. the final figure is, of course, multiplied by two.

9. Carbohydrate Tolerance Test (Killian[3]).—Principle.—Blood sugar is determined at hourly periods following the ingestion of 1.75 gm. of glucose per kilogram of body weight. Urinary sugar for the 24 hour period following the ingestion of the glucose is also determined.

Procedure.—Give the patient, the first thing in the morning, a standard breakfast consisting of two slices of bread, one egg in any form and one cup of water. Two hours after this breakfast have the patient empty the bladder and then drink 200 c.c. of water. One hour later collect a specimen of urine and one of blood to serve as controls. Then give the patient 1.75 gm of glucose per kilo of body weight. The glucose is given in 50 per cent solutions.[4] Collect 3 or 4 specimens of blood at hourly intervals and analyze for sugar. Following the taking of glucose collect a 24 hour specimen of urine and determine its sugar content.

[1] Occasionally the final filtrates in this or other picric acid methods develop a little turbidity during heating. Unless such turbidity is fairly marked it is of no account. When desired, the final colored solution may be filtered through a small folded filter into the colorimeter cup.

[2] *Permanent Standards.*—The picramic acid standard is best prepared from a stock solution containing 100 mg. of picramic acid and 200 mg. of sodium carbonate per liter. One hundred twenty-six c.c. of this solution are treated with 1 c.c. of the 20 per cent sodium carbonate solution and 15 c.c. of the picrate-picric acid solution, and diluted to 300 c.c. with distilled water. This solution matches exactly the color obtained by treating 0.64 mg. of glucose, as in the above method and diluting to 12.5 c.c. A satisfactory preparation of picramic acid may be obtained from the J. T. Baker Chemical Co., Phillipsburg, N. J.

The standard prepared from potassium dichromate contains 800 mg. of pure potassium dichromate in a liter of water.

[3] Killian: *Proc. Soc. Exper. Biol. and Med.*, 17, 91, 1920.

[4] Janney and Isaacson (*Arch. Int. Med.*, 22, 160, 1918) administer the glucose in 40 per cent solution together with the juice of one lemon and without the preliminary standard meal. Two blood specimens are taken—just before giving the glucose and at the end of two hours.

Interpretation.—In normal individuals blood sugar rises from the normal value of about 100 mg. to about 150 mg. per 100 c.c. at the end of the first hour and returns to normal by the end of the second hourly period. In pathological conditions the curve does not follow the normal course. Hyperthyroidism, diabetes mellitus, and nephritis show much greater values, depending on the severity of the disease, and the return to normal is delayed for 3 hours or more. The high sugar concentration in the blood during the test may or may not be accompanied by glycuresis, depending upon the "threshold point" of the kidney. In

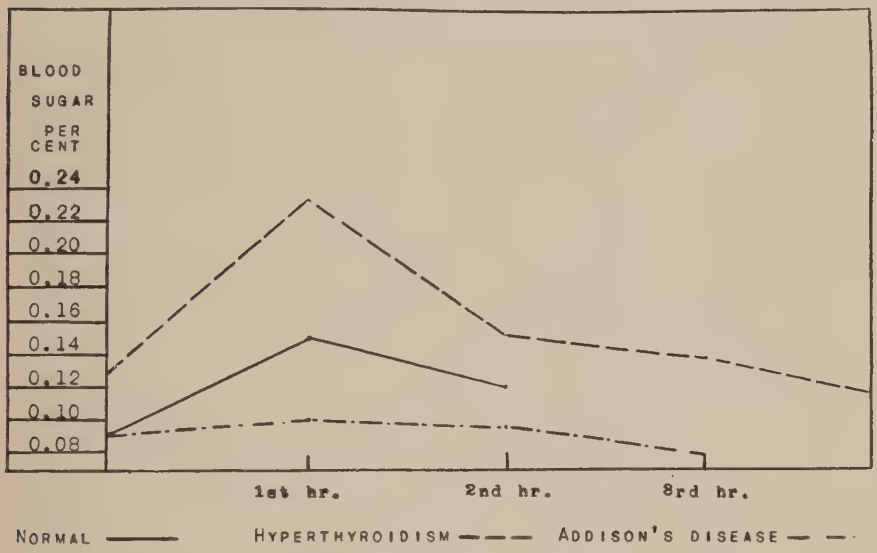

FIG. 120.—SUGAR TOLERANCE CURVES.
(Myers: "Practical Chemical Analysis of the Blood," 1924).

diabetes the threshold point is usually, and sometimes markedly, increased above its normal range of 160 to 180 mg. per 100 c.c. In hypo-endocrine conditions, in which the blood sugar is low ordinarily, the curve of blood sugar during a tolerance test is quite "flat." Curves obtained by Killian in hyperthyroidism and Addison's disease, together with the curve of a normal case are shown in Fig. 120,[1] and the curve of a diabetic is shown in Fig. 121. Wherever possible, it is better practice to obtain quarter-hour specimens of blood, during the first hour. This provides more information concerning the general nature of the curve and the height of the peak. John[2] believes that a diagnosis of diabetes is justified if the curve remains high after 3 hours, irrespective of the height to which it has risen. Petty and Stoner[3] on the other hand

[1] Myers: Practical Chemical Analysis of Blood, C. V. Mosby Company, 1924.
[2] John: *Am. J. Med. Sci.*, 169, 102, 1925; *J. Metab. Res.*, 1, 497, 1922.
[3] Petty and Stoner, *Am. J. Med. Sci.*, 171, 842, 1926.

regard the criterion as a rise above 180 mg. They base their claim on simultaneous determinations of respiratory quotients, which show very slight rise in diabetics, but follow the sugar curves in normal individuals and in renal glycosurics. In non-diabetics the peak of the curve is usually between ½ and 1 hour, while that of diabetics is nearer 2 hours.

In studying the glucose tolerance of an individual, attention should be directed to certain factors which influence it. The effect of fasting and of previous diet on tolerance of rabbits for glucose are illustrated

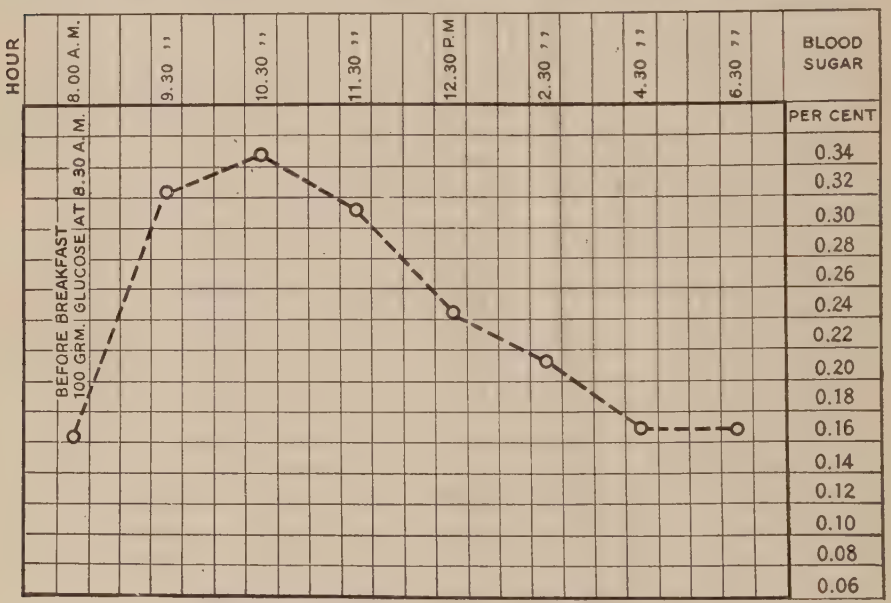

FIG. 121.—BLOOD SUGAR CURVE OF DIABETIC AFTER GLUCOSE INGESTION.
(Martin and Mason: *American Journal of Medical Sciences*, 153, 50, 1917.)

in Figs. 271 and 272 in Chapter XXXII. It has been shown by Foster that carbohydrate tolerance tests conducted on finger blood, which is practically arterial, give higher and sharper curves than venous blood collected simultaneously. It appears that some of the glucose of arterial blood is transformed to glycogen in muscle tissue. The glycogen synthesis appears to be stimulated to overactivity since the blood sugar curve falls below its initial level. For further discussion of the application of sugar tolerance tests consult papers by Hamman and Hirschman,[1] Janney and Isaacson, Bailey, Williams and Humphreys, Allen, Stillman and Fitz, Killian, Macleod, Foster, and du Vigneaud and Karr.

[1] Hamman and Hirschman: *Arch. Int. Med.*, 20, 761, 1917; Bailey: *Arch. Int. Med.*, 23, 455, 1919; Williams and Humphreys: *Arch. Med. Int.*, 23, 537, 546, 559, 1919; Allen, Stillman, and Fitz: Monograph of the Rockefeller Institute for Medical Research, No. 11, 1919; Macleod: *Physiological Reviews*, 1, 208, 1921; Foster: *J. Biol. Chem.*, 55, 291, 303, 1923; du Vigneaud and Karr: *J. Biol. Chem.*, 66, 281, 1925.

9A. Carbohydrate Tolerance Test (Improved).[1] **Principle.**—The former technique is modified in order to furnish information as to the actual amount of sugar absorbed by removal of unabsorbed sugar by means of a Rehfuss tube, and to correct for changes in blood volume as determined by hemoglobin estimations.

Procedure.—A fasting subject is given to drink a known amount of glucose dissolved in water. The ingested solution contains not more than 20 per cent of glucose in order to secure good absorption and the volume is limited to 500 c.c. since it is difficult for most persons to drink rapidly a larger quantity of fluid. An hour later the stomach is emptied as completely as possible and the amount of recovered sugar is determined. The difference between the amount recovered and the amount ingested measures, with a small error, the amount absorbed. This is expressed as grams of sugar absorbed for each kilogram of the subject's weight for each hour of time. Blood sugar and hemoglobin determinations are made at the time the sugar is ingested, when the stomach is emptied and at later intervals.

Since the amount of absorbed sugar and the resultant hyperglycemia vary in each case an attempt is made to make the results in one case comparable to others in the following way. The blood sugar readings are corrected for any demonstrable blood volume change and for the variable amounts of sugar which have been absorbed. The first correction is made from any changes found in the hemoglobin percentage. The second correction is made from the amount of sugar absorbed and the degree of glycemia found. The results are made uniform by estimating from these data the proportional blood sugar readings which would have been obtained under the conditions outlined had 0.800 gram of glucose for each kilogram of weight of each subject been absorbed. This dosage of glucose was selected because Wilder and Sansum found that it represented the upper limit of normal glucose tolerance for an hour by the intravenous method in human subjects.

Interpretation.—See above.

10. Determination of Cholesterol (Method of Myers and Wardell Modified[2]**).—Principle.**—The blood is dried on plaster of Paris and extracted with chloroform. Cholesterol is determined colorimetrically after adding to the chloroform extract acetic anhydride and sulphuric acid.

Procedure.—For the determination, 1 c.c. of blood, plasma or serum is pipetted into a porcelain crucible or small beaker containing 4 to 5 gm. of plaster of Paris, stirred, and dried, preferably in a drying oven for an hour. It is now emptied into a small paper extraction shell (4 cm. long) and then inserted in a short glass tube (2.5 × 7 cm.) in the bottom and sides of which are a number of small holes. This is now attached to a large cork on a small reflux condenser and the tube and cork inserted in the neck of a 150 c.c. extraction flask containing about 20 to 25 c.c. of chloroform. Extraction is continued for 90 minutes on an

[1] Beeler, Bryan, Cathcart and Fitz: *Jour. Metab. Res.*, 1, 549, 1922.
Oser and Karr: *Arch. Int. Med.*, 36, 507, 1925.
Myers and Wardell: *Jour. Biol. Chem.*, 36, 147, 1918.

electric hot plate, the chloroform made up to some suitable volume, such as 25 c.c., filtered if necessary, and colorimetric estimation carried out as follows: 10 c.c. of the chloroform extract are pipetted into a dry test tube, and 2 c.c. of acetic anhydride and 0.2 c.c. of concentrated sulphuric acid are added. 10 c.c. of a standard solution of cholesterol[1] in chloroform are treated in exactly the same way. After thorough mixing, the solutions are placed in the same light by which the colorimetric readings are to be made for exactly 15 minutes[2] to allow the color to develop. If the Duboscq colorimeter is used, it is necessary that the cups should be remounted in plaster of Paris instead of balsam.

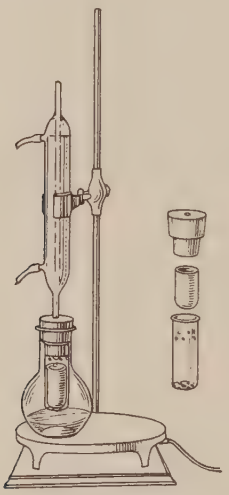

FIG. 122.

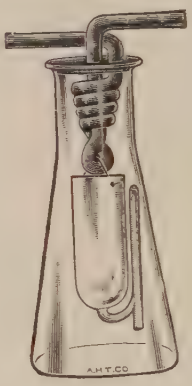

FIG. 123.

EXTRACTION APPARATUS FOR CHOLESTEROL DETERMINATION.

(Fig. 122 after Myers: "Practical Chemical Analysis of Blood," C. V. Mosby Co., St. Louis, 2nd Ed., 1924).

The apparatus illustrated in Fig. 123 is very neat and compact and has been used successfully for cholesterol extractions.[3] The extraction thimble is inserted in the siphon cup (perforated procelain cup may be used), chloroform introduced into the flask (pyrex) and the extraction conducted while water flows through the condensing coil.

Calculation.—

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.8 \times \frac{100}{0.4} = \text{mg. cholesterol per 100 c.c. blood.}$$

Set the unknown on the left at 20; the cholesterol content is then given by 10 times the reading of the standard.

[1] Prepare a stock solution by dissolving 0.160 gm. of pure cholesterol in 100 c.c. of redistilled chloroform. The working standard is made by diluting 5 c.c. of the stock standard to 100 c.c. with chloroform. 10 c.c. = 0.8 mg. cholesterol.

[2] In order to get the proper temperature for color development in warm weather it is advisable either to keep the reagents in a cool place or to insert the tubes in water during the development of the color.

[3] This apparatus, while designed for rubber analyses, was first suggested for use in cholesterol determinations by Dr. W. H. Stoner, of the Philadelphia General Hospital. It may be obtained from A. H. Thomas Co., Philadelphia

Interpretation.—Normal blood serum contains from 150 to 190 mg. per cent of cholesterol and whole blood about 140 to 180 mg. per cent. Values of from 170 to 350 have been noted in chronic and acute nephritis. High cholesterol values are of diagnostic significance in chronic nephrosis (see p. 357). In diabetes, values of 150 to 300, and in lipemia extremely high values (up to 3600) are found. The hypercholesterolemia of diabetes is reduced by insulin. An increase is also found in pregnancy. In cholelithiasis high values are frequently obtained. In pernicious anemia values as low as 70 mg. per cent are noted. Cholesterol is increased by a high lipoid diet and decreased by a diet low in cholesterol.

11. Determination of Fatty Acids and Cholesterol. Method of Bloor, Pelkan, and Allen.[1]**—Principle.**—The lipoids are extracted from blood plasma by means of hot alcohol-ether. The extract is saponified, the cholesterol extracted with chloroform, and the soaps with hot alcohol. Cholesterol is then determined colorimetrically and fatty acids by nephelometric estimation of the cloud formed on acidification of the soap solution.

Procedure.—Extraction and Saponification.—Five c.c. of blood plasma are measured into a 100 c.c. flask containing about 75 c.c. of a mixture of 3 parts alcohol and 1 part ether (both redistilled). The plasma is made to enter in a slow stream of drops and the liquid in the flask is kept rotating rapidly to prevent the formation of large aggregates of precipitate. At once, or after standing till a convenient time, the flask is immersed in boiling water with frequent and strong rotation (to prevent superheating) until the liquid begins to boil, then cooled to room temperature, made up to volume, mixed, and filtered. For the determination a volume (10 to 20 c.c.), containing about 2 mg. of fatty acid, is measured into a small Erlenmeyer flask (50 to 100 c.c.) of Non-sol glass (Pyrex is less suitable because it is readily attacked by the strong alkali), 0.1 c.c. of concentrated NaOH made from sodium[2] is added and the mixture evaporated on the water bath. When the

[1] Bloor, Pelkan and Allen: *Jour. Biol. Chem.*, 52, 191, 1922.

[2] Solutions required for fat and cholesterol determinations:

Standard Fat Solutions.—A 95 per cent alcoholic solution of palmitic and oleic acids of which 5 c.c. contain 2 mg. of a mixture consisting of 60 per cent oleic acid and 40 per cent palmitic acid. The standard is conveniently made by first preparing solutions of oleic and palmitic acids, each containing 200 mg. of fatty acid in 500 c.c. of alcohol. For use 60 c.c. of the oleic acid solution are mixed with 40 c.c. of the palmitic acid solution.

Standard Cholesterol Solution.—This is a solution of cholesterol in chloroform containing from 0.5 to 1 mg of cholesterol in 5 c.c., depending on the cholesterol content of the blood which is being measured. For most purposes a standard containing 0.50 mg. of cholesterol in 5 c.c. of solution will be found suitable. For convenience in weighing the cholesterol, a standard twenty times the strength of the final standard is prepared and this is diluted as needed.

Sodium Hydroxide.—Made from metallic sodium by exposing the metal, in a closed vessel containing distilled water at room temperature, over a receiver to catch the hydroxide which drips off the metal. The action is slow but the apparatus requires little attention. A strong pure hydroxide is obtained. A desiccator containing water instead of sulphuric acid may be used. On the porcelain plate a round bottom crystallizing dish is set in which is placed an inverted watch glass fitting about half way down the sides of the dish. The sodium is placed in the center of the watch glass, and hydroxide drips from it into the crystallizing dish.

Chloroform.—The chloroform used must be neutral in reaction and free from moisture and alcohol.

volume of liquid has been reduced to a few drops the flask should be rotated or shaken occasionally so as to distribute the liquid evenly over the bottom (but not over the sides). The drying is then continued until only 2 or 3 drops of liquid remain and the odor of alcohol is entirely gone. The alkali is then *partially* neutralized by the addition of 0.1 c.c. of dilute sulfuric acid (1 volume concentrated acid, 3 volumes water), and the liquid well mixed and distributed over the bottom of the flask as before. The drying is then continued on the water bath until the residue is dry and all the moisture has disappeared from the sides of the flask. The process of drying is a very important step in the method, since the separation is not quantitative if the drying is either carried too far (in which case some of the cholesterol cannot be recovered by the cold treatment), or not far enough (when a part of the soap or fatty acids is extracted with the cholesterol). The amount of acid added should be somewhat less then enough to neutralize the alkali since otherwise fatty acids would be set free and dissolve in the chloroform. For the same reason the added acid should be well mixed with the residue in the flask so as to insure its complete neutralization. If there is not enough liquid in the flask to allow complete mixing, a drop or two of distilled water should be added. The reason for the addition of acid is twofold, first to prevent destruction of cholesterol by the strong alkali, and second by the formation of the crystalline sodium sulfate the residue is made porous so that the solvents penetrate readily. The heating should be carried through all its stages on a water bath and not on an electric hot-plate since it has been found impossible to prevent overheating on the latter.

Separation and Determination of Cholesterol.[1]—After cooling, 10 c.c. of chloroform are added and the flask is allowed to stand for ten minutes; it may be shaken occasionally so that the solvent may reach any material adherent to the sides. The chloroform extract is poured through a 5½ cm. hardened filter into another small flask and the extraction twice repeated with 5 c.c. of chloroform. If the drying and distribution of the salt have been carefully carried out, very little of the salt will break loose from the bottom of the flask during the chloroform extraction and the fatty acids are quantitatively retained. The combined chloroform extract is then evaporated down to 2 or 3 c.c., poured into a 10 c.c. glass-stoppered, graduated cylinder, made up to 5 c.c. with chloroform washings from the flask, and the cholesterol then determined by the use of the Liebermann-Burchard reaction as follows: To the contents of the graduated cylinder made up to 5 c.c. are added 1 c.c. of acetic anhydride and 0.1 c.c. of pure concentrated sulfuric acid, the cylinder is stoppered, and the whole well mixed. The cylinder is allowed to stand for 15 minutes at a temperature of 20–22°C., exposed to the same light by which readings are later to be made. (The color is sensitive to light and this precaution is necessary in order to avoid changes in tint during the reading.) It is then transferred to the colorimeter cup and compared with a suitable standard similarly prepared from pure cholesterol. The standard cholesterol for this purpose should contain ordinarily 0.5 mg. of cholesterol in 5 c.c. of choloform.

Determination of Fatty Acids.—The residue in the small flasks after extraction with chloroform as above is treated with boiling alcohol for the extraction of the fatty acids (in the form of soaps) in the following way: 10 c.c. of redistilled alcohol are added to each flask, the mixture is raised to boiling on an electric stove or water

[1] Sackett, *Jour. Biol. Chem.*, 64, 203, 1925, describes a micro-modification of Bloor's method for cholesterol which economizes material and time. The appearance of a brownish tinge in the unknown, so frequent in Bloor's method, is eliminated by extracting without heat.

bath and kept boiling very gently for a period of ten minutes. The hot alcohol is then poured through the small hardened filter which was used in filtering the chloroform, into a 100 c.c. Erlenmeyer flask. The extraction with alcohol is repeated once, using 5 c.c. of alcohol, the hot extracting fluid being poured through the filter into the flask. The combined filtrates are evaporated to a small volume, about 2 to 3 c.c., then transferred quantitatively to a small graduated, glass-stoppered cylinder, and the flask is rinsed out with just enough alcohol to bring the volume in the cylinder up to 5 c.c. One hundred c.c. of distilled water are next measured into a 200 c.c. beaker and the alcoholic extract of the fatty acid is added, with stirring, through a small funnel with the stem drawn out to form an opening about 1 mm. in diameter and extending nearly to the bottom of the beaker. The cylinder is rinsed once with the solution in the beaker and the rinsings are poured back into the beaker through the funnel. To another beaker containing 100 c c. of water are added through a pipette with stirring 5 c.c. of the alcoholic standard containing 2 mg. of a mixture of oleic and palmitic acids containing 60 per cent oleic and 40 per cent palmitic acids in 95 per cent alcohol (redistilled). Ten c.c. of dilute hydrochloric acid (1 part concentrated acid, 3 parts water) are added to each beaker with stirring, and after standing not less than 3 nor more than 10 minutes the solutions are compared in the nephelometer.

12. Determination of Lipoid Phosphorus (Lecithin). Methods of Bloor[1] and Benedict and Theis.[2] Principle.—The extracted lipoids are oxidized by nitric-sulphuric acid mixture, and the lipoid phosphoric acid determined by a colorimetric method.

Procedure.—Introduce 1 c.c. of well mixed blood into 20 c.c. of a mixture of 3 parts alcohol and 1 part ether (both redistilled) contained in a 25 c.c. volumetric flask. The blood is made to enter slowly and the mixture in the flask kept rotating so as to prevent the formation of large aggregates of precipitate which are difficult to extract. The flask is then immersed in boiling water with frequent and vigorous rotation (to avoid superheating), until boiling just commences. It is then cooled to room temperature, made up to 25 c.c. with alcohol-ether, mixed and filtered. For the oxidation, 5 c.c. of the filtrate are introduced into a Pyrex test tube (20 × 175 mm.) graduated at 5 c.c., which is then immersed in a boiling water bath, and evaporated to dryness. 0.5 c.c. of a mixture of equal parts of concentrated nitric and sulphuric acids are added and mixed well with the residue. The digestion is conducted over a microburner with precautions against overheating. Water and oxides of nitrogen pass off at first, followed by sulphuric anhydride fumes. The flame is reduced and heating continued until the brown fumes just reappear. After cooling two minutes, a drop or two of 1 per cent sucrose solution is introduced so as to produce a charred mixture. On heating again the solution should again become clear. (If a brown or yellow color persists after half a minute of boiling, a trace of nitric acid should be added and the boiling resumed.) Cool and add 2 c.c. of distilled water, rinsing down the sides of the tube. Neutralize approximately by adding carefully a previously determined amount (about 6 or 7 drops) of concentrated CO_2-free NaOH. Cool and dilute

[1] Bloor: *Jour. Biol. Chem.*, 36, 33, 1918. See also Oser and Karr, *Arch. Int. Med.*, 36, 507, 1925.

[2] Benedict and Theis: *Jour. Biol. Chem.*, 61, 63, 1924.

with distilled water to the 5 c.c. mark. Proceed as directed under the Benedict and Theis method for inorganic phosphorus (p. 406), using the same standard phosphorus solution.

Calculation.

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.025 \times \frac{100}{0.2} = \text{mg. lipoid phosphorus per 100 c.c. blood.}$$

By setting the unknown at the left at 12.5 mm., the result is given directly by the reading of the standard. "Lecithin" contains about 4.0 per cent phosphorus.

Interpretation.—According to Bloor lecithin is an intermediate step in the metabolism of fats. Absorbed fat in the plasma is said to be taken up by the corpuscles and there transformed into lecithin. High lipoid phosphorus values in corpuscles are frequently observed in anemia, showing possibly an extra strain on this function of the corpuscles. Normal whole blood contains about 12 to 14 mg. lipoid phosphorus per 100 c.c., about 50 to 60 per cent of which is contained in the corpuscles. Pathologically greater variations are noted in the lecithin of corpuscles. In diabetes and nephritis, the lipoid phosphorus follows the degree of lipemia. Increases in lecithin are also noted in pregnancy and in certain hepatic conditions. The existence of a constant balance between cholesterol (which is antihemolytic) and lecithin (hemolytic) has not been definitely established.[1]

13. Determination of Bile Pigment in Serum. Icteric Index (Meulengracht).[2] **Principle.**—The intensity of yellow pigmentation of serum is compared with a standard potassium bichromate solution.

Procedure.—Separate the serum from 4 or 5 c.c. of freshly drawn, unhemolyzed blood. According to its depth of color, dilute 1 c.c. with 0.9 per cent sodium chloride in a graduated cylinder until an approximate match with the standard potassium bichromate solution[3] is obtained. Compare these solutions in a colorimeter. The Hellige-Leitz or Bock-Benedict instruments are well suited to the use of small amounts of liquid.

Calculation.

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \text{Dilution} = \text{Icterus Index.}$$

For example, if one c.c. of serum is diluted to 25 c.c. and the standard reads 17.5 when the unknown is set at 20, the icterus index is 21.9.

Interpretation.—See under Van den Bergh Test.

13A. Van den Bergh Test.[4] **Principle.**—Serum is treated with a diazotizing reagent and the red color which results from its reaction

[1] Oser and Karr: *Arch. Int. Med.*, 36, 507, 1925.

[2] Meulengracht: *Deutsch. Arch. f. klin. Med.*, 132, 285, 1920. For colorimetric method for *bile salts* in blood see Szilard: *Bioch. Zeit.*, 173, 440, 1926.

[3] A 0.01 per cent potassium bichromate solution, to which is added 2 drops of concentrated H_2SO_4 per 500 c.c. This standard should be kept in a brown glass bottle or in the dark. Preston (*J. Lab. Clin. Med.*, 11, 879, 1926) advocates the use of a colored glass ("Uran") disc as a standard (Klett Manufacturing Co., New York, N. Y.).

[4] Van den Bergh: *Presse Medical*, 29, 441, 1921.
McNee: *Quart. Jour. Med.*, 16, 390, 1923.

with bilirubin is taken as an index of the type and extent of bilirubinemia, depending on the rate of appearance and depth of color.

Procedure.—Separate the serum from 4 or 5 c.c. of freshly drawn, unhemolyzed blood, into a clean test tube. Direct Reaction.—To one c.c. of serum add 2 c.c. of distilled water and 0.5 c.c. of the diazo reagent.[1] A control may be run by adding, in another tube, slightly acidified distilled water to the diluted serum, in place of the diazo reagent. The positive direct reaction may be one of three types: immediate, if the color change begins within 30 seconds and reaches its maximum within a minute or so; delayed, if the color change begins after 30 seconds and requires several minutes (perhaps as many as 15 or 20) to reach its maximum; or biphasic, if the color appears promptly but requires some minutes to become maximal. The last reaction is a combination of the first two.

Indirect Reaction.—To one c.c. of serum in a clean centrifuge tube, add 2 c.c. of 96 per cent alcohol. Mix and centrifuge for 5 minutes. Separate the protein-free supernatant liquid, and to one c.c. add 0.5 c.c. of alcohol and 0.25 c.c. of the diazo reagent. Allow to stand a few minutes and compare with a standard[2] in a colorimeter such as referred to under Icteric Index.

Calculation.—In the indirect reaction, no bilirubin is regarded as lost by precipitation of the protein. Making allowance for the volume of precipitate and the dilution, it is assumed that in the final solution the bilirubin is diluted 5 times. If the color matches the standard, the serum, therefore, contains 5 units of bilirubin. The colorimetric calculation is

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 5 = \text{units of bilirubin.}$$

A unit of bilirubin is 1 part in 200,000, or a concentration of 0.5 mg. per 100 c.c. of serum.

Interpretation.—The normal icterus index ranges between 4 and 6 the zone of latent jaundice (*i.e.*, hyperbilirubinemia without clinical signs of jaundice) is between 6 and 15. Above this value icteric symptoms may be observed. According to the quantitative (indirect) van den Bergh test, normal serum contains between 0.2 and 0.5 units (0.1

[1] Solution A. To 1 gm. sulphanilic acid add 15 c.c. concentrated hydrochloric acid. Dissolve and dilute to 1000 c.c. with distilled water. Solution B. 0.5 per cent sodium nitrite. Just before the test, prepare the diazo reagent by mixing 25 c.c. of solution A with 0.75 c.c. of solution B.

[2] The standard recommended by Van den Bergh is an ethereal solution of ferric thiocyanate prepared as follows:

Stock ferric alum solution: Dissolve 0.1508 gm. ferric ammonium sulphate in 50 c.c. conc. hydrochloric acid and dilute with water to 100 c.c. Keeps indefinitely.

Standard ferric alum solution: To 10 c.c. of the above add 25 c.c. conc. hydrochloric acid and dilute to 250 c.c. Keeps about 6 months.

Potassium thiocyanate solution: 20 per cent.

Standard colorimetric solution: Into a small separatory funnel place 3 c.c. of the standard ferric alum solution and 3 c.c. of the potassium thiocyanate. Extract all the red color by mixing gently with 12 c.c. of ether. Drain off the lower aqueous layer. The ethereal thiocyanate solution is used as the standard and should be prepared fresh daily. It represents a 1:200,000 dilution of azo-bilirubin, *i.e.*, one unit of bilirubin.

McNee (in a lecture at the College of Physicians, Philadelphia) recommended the use of a cobalt sulphate solution as the standard, instead of the ethereal thiocyanate solution. This has the advantage of simplicity in preparation and greater permanence, being an aqueous solution. It is prepared by dissolving 2.161 gm. of anhydrous cobalt sulphate, or 3.92 gm. of $CoSO_4 \cdot 7H_2O$, in distilled water and diluting to 100 c.c.

to 0.25 mg. per 100 c.c.) of bilirubin. Assuming that this range corresponds to the normal icterus index, the zone of latent jaundice is between 0.5 and 2.0 units (0.25 to 1.0 mg. per 100 c.c.) of bilirubin.

Hyperbilirubinemia may occur in diseases of the liver or biliary tract, and also in extra-hepatic conditions of a hemolytic nature, such as those accompanying infectious diseases, pernicious anemia, hemolytic anemia, rupture of blood vessels, etc. Low values for bilirubin may be found in secondary anemia (icterus index between 2 and 4). The determination is, therefore, of importance in the differential diagnosis of anemias. The renal threshold for bilirubin is 3.5–4.0 units in the serum.

The three types of direct van den Bergh reaction are thought to be due to some chemical or physicochemical differences in the bilirubin which are attributed to the path by which the pigment enters the serum. In cases of frankly obstructive jaundice the immediate direct reaction is obtained. The direct reaction is delayed in those forms of jaundice resulting from hemolytic processes. The biphasic reaction indicates that both processes, obstruction and hemolysis, are probably at work, and often occurs in cases of liver cell damage. The cause of the variation in rate of reaction and the question of its diagnostic significance have been the subject of considerable investigation. The colloidal and chemical nature of the serum protein, in particular globulin, is probably intimately concerned.[1] The van den Bergh test has won considerable favor among clinicians.[2] An extensive study of the icterus index has been made by Bernheim.[3] Other pigments of serum have been shown not to be present in sufficient concentration to interfere with the test for icterus index. The chief exception, however, is the increase in carotin which occurs after the ingestion of carrots, which should, therefore, be avoided.

14. Determination of Blood Proteins.[4] **Principle.**—*Total proteins* are determined in serum or plasma by a micro-Kjeldahl method employing direct nesslerization, making the appropriate correction for non-protein nitrogen. *Fibrinogen* in plasma is estimated from the difference between total proteins and the protein content of the filtrate

[1] Adler and Strauss: *Zeit. f.d. ges. exp. Med.*, 44, 1, 1924. de Micheli and Greppi, *Arch. patol. clin. med.*, 2, 58, 1923. Fiessinger and Casteran: *Compt. rend. soc. biol.*, 92: 71, 1925.

[2] McNee: *Quart. Jour. Med.*, 16, 390, 1923.
Andrews: *Ibid.*, 18, 19, 1924.
Brown, Ames, Warren and Peabody: *Jour. Clin. Invest.*, 1, 295, 1925.
McNee and Keefer: *Brit. Med. Jour.*, 2, 52, 1925.

[3] Bernheim: *Jour. Am. Med. Assn.*, 82, 291, 1924; *Arch. Path. Lab. Med.*, 1, 747, 1926.

[4] Adapted in the Biochemical Laboratories of the Philadelphia General Hospital, combining the methods of Howe, *Jour. Biol. Chem.*, 49, 109, 1921, Wu, *Jour. Biol. Chem.*, 51, 33, 1922, and Koch and McMeekin: *Jour. Am. Chem. Soc.*, 46, 2066, 1924 (see p. 370). The paper of Howe describes methods for the fractionation of the various types of globulin occurring in serum. Wu's method is based on the determination of the tyrosine content of each protein fraction and calculation of the proteins from their tyrosine equivalents which vary with the species of animal.

obtained after precipitation of fibrinogen by calcium chloride. *Albumin* is determined on the filtrate obtained after precipitation of the globulin by 22.2 per cent sodium sulphate at 37°C. (Howe's method). *Globulin* in serum is estimated by subtracting albumin from total protein; in plasma by subtracting albumin and fibrinogen from total protein.

Procedure. Total Proteins.—Dilute exactly one c.c. of the serum or plasma to 50 c.c. in a volumetric flask with 0.9 per cent sodium chloride. Using one c.c. of this solution, proceed as directed under "Digestion." Fibrinogen.—To one c.c. of plasma in a 50 c.c. graduated cylinder add 48 c.c. of 0.9 per cent sodium chloride and one c.c. of 2.5 per cent calcium chloride. Mix and let stand 20 minutes. The fibrin clot may be broken with a glass rod and the solution filtered, or the clot may be wrapped around the glass rod by rotation, leaving a clear solution. One c.c. of the latter is used in the digestion. Albumin.—To one c.c. of serum or plasma in a 50 c.c. graduated cylinder add exactly 30 c.c. of 22.2 per cent sodium sulphate which has been warmed to 37°C.[1] Stopper the cylinder and let stand at that temperature for 3 hours. Filter through a fine grade of filter paper (Schleicher and Schull or Munktell) into a test tube, keeping the funnel covered with a watch glass during the filtration. The filtrate should be perfectly clear, otherwise return to filter paper until it is. Use one c.c. of this filtrate and proceed as directed under "Digestion."

Digestion.—Into a Pyrex test tube graduated at 35 and 50 c.c. (see method for non-protein nitrogen) introduce one c.c. of the solution prepared as above directed and 1 c.c. of sulphuric acid digestion mixture.[2] Add a few chips of broken porcelain and one drop of caprylic alcohol. Heat with a microburner (preferably in a hood) until all the water boils off and white fumes appear. Insert a small funnel in the tube and reduce the flame. Continue heating until the material chars and dense white fumes fill the tube. Cool for 30 seconds and then add carefully, a drop at a time, 30 per cent hydrogen peroxide until the solution clears up, recording the number of drops used. Boil again for 1 minute. Cool and dilute to 35 c.c. with distilled water. Into a 50 c.c. graduated cylinder introduce 5 c.c. of standard ammonium sulphate solution[3] and one c.c. of the acid digestion mixture. Dilute this standard to 35 c.c. To the standard and the unknown now add 15 c.c. of Nessler's solution and compare in the colorimeter.

A blank determination should be carried out on the hydrogen peroxide. To one c.c. of the boiling digestion mixture in a Pyrex tube add drop by drop exactly 30 drops of the peroxide. After the digeston is completed, dilute, nesslerize and compare as above. Obtain the N equivalent of one drop of the peroxide.

Calculation.—The protein values are calculated from the nitrogen contents by multiplying by 6.25. A correction for the nitrogen content of the peroxide is made

[1] Dissolve 22.2 grams of anhydrous sodium sulphate in distilled water and make up to 100 c.c. in a volumetric flask, keeping the temperature at 37°. It is convenient to prepare this solution in an incubator room.

[2] To 10 c.c. of 5 per cent copper sulphate solution add 100 c.c. of concentrated sulphuric acid. Carefully pour this mixture into 100 c.c. distilled water, stirring constantly.

[3] Dissolve and dilute 0.1414 gm. c.p. ammonium sulphate to 1000 c.c. with distilled water, (5 c.c. = 0.15 mg. N.)

by subtracting from the total nitrogen in the sample, the nitrogen equivalent per drop (E) times the number of drops used (n).

$$\text{Per cent Protein} = \left[\left(\frac{R_s}{R_u} \times 0.15 - nE\right)\frac{100}{V} - \text{NPN}\right]\frac{6.25}{1000}$$

in which R_s and R_u represent the readings of the standard and the unknown respectively; n and E have the significance above described; V represents the c.c. of serum or plasma used in the determination; NPN represents the non-protein nitrogen in mg. per 100 c.c. (see methods 2 and 2A, on pages 368 and 370). For total protein, V = 0.02; for protein in the fibrinogen filtrate, V = 0.02; for albumin, V = 0.0323 $\left(\text{i.e. } \frac{1}{31}\right)$. Total protein and albumin are determined directly.

Fibrinogen = Total protein − Protein in fibrinogen filtrate.

Globulin = Total protein − Albumin − Fibrinogen, in the case of plasma. Since fibrinogen is absent from serum, the latter factor is omitted in the calculation of serum globulin.

15. Determination of Phenols. Method of Theis and Benedict.[1] **Principle.**—Phenols react with diazotized *p*-nitroaniline to form orange colored compounds. The estimation is colorimetric, and is not applicable to the urine.

Procedure.—Precipitate blood proteins as in the Folin-Wu procedure but use 2 volumes of water instead of 7. To 10 c.c. of the 1:5 filtrate add 1 c.c. of 1 per cent gum acacia solution, 1 c.c. of 50 per cent sodium acetate solution and 1 c.c. of the diazotized nitroaniline reagent.[2] After 1 minute add 2 c.c. of 20 per cent sodium carbonate solution. Compare with a similarly treated phenol solution containing 0.025 mg. of phenol in 10 c.c. For preparation of standard see p. 766. To determine total phenols (free and conjugated) treat 10 c.c. of the blood filtrate in a test tube with 0.25 c.c. concentrated HCl and heat for 10 minutes in a boiling water bath. Cool. Neutralize with NaOH. Add same amounts of acid and alkali also to standard and unheated solutions. Complete determination as before. Blood contains from 1–2 mg. of free phenols per 100 c.c. Conjugated phenols may amount to 0.1–0.2 mg.

16. Determination of Lactic Acid. Method of Clausen.[3] **Principle.** The glucose of blood filtrates is removed with copper sulphate and calcium hydroxide, the lactic acid converted to acetaldehyde and titrated as in the method for urine (p. 743).

Procedure.—To 10 c.c. of Folin-Wu filtrate representing 1 c.c. of blood add 2 c.c. 10 per cent $CuSO_4$ and 2 c.c. of 5 per cent suspension of $Ca(OH)_2$. Shake at intervals for one-half hour and centrifuge. Of the filtrate 5 c.c. duplicates are taken for analysis as in the method for urine p. 743. For the blank treat 10 c.c. of 0.1 per cent glucose in the same manner as blood filtrate.

[1] Theis and Benedict: *Jour. Biol. Chem.*, 61, 67, 1924. See also Rakestraw: *Jour. Biol. Chem.*, 56, 109, 1923.

[2] Dissolve 1.5 gm. of *p*-nitroaniline base in 500 c.c. of water with 40 c.c. of concentrated hydrochloric acid. The diazotized reagent is prepared fresh daily by adding to 25 c.c. of this solution, 0.75 c.c. of 10 per cent sodium nitrite solution.

[3] Clausen: *Jour. Biol. Chem.*, 52, 263, 1922.

Interpretation.—Blood of normal individuals in the resting state contains 5–20 mg. of lactic acid per 100 c.c. During severe exercise this may rise as high as 200 mg. A rise is also observed in eclampsia.[1]

16A. Determination of Lactic Acid in Tissues.[2] **Principle.**—Protein is removed by precipitation with mercuric chloride. The analysis is then carried out as on blood filtrates.

Procedure.—Kill treated and control animals practically simultaneously by a blow on the head. Remove tissues promptly or freeze *in situ* in a stream of compressed CO_2 before removal. Weigh, transfer to a cold mortar and cut up and rub to a fine suspension with ice cold 2 per cent HCl. Transfer quantitatively to a small volumetric flask (usually 10 c.c.) and add 5 per cent $HgCl_2$ to the mark. Let stand 1 to 3 hours. Filter. Precipitate the mercury with H_2S and remove the latter in the usual way. Neutralize 5 c.c. of the final filtrate carefully to litmus paper and make up to 10 c.c. Add 2 c.c. of 10 per cent $CuSO_4$ and 2 c.c. of a 5 per cent suspension of $Ca(OH)_2$. Shake occasionally during half an hour, centrifuge and take two 5 c.c. portions for the lactic acid determination according to Clausen (p. 400). For discussion see p. 562.

17. Determination of Chlorides (Whitehorn).[3]—*Principle.*—The chlorides are precipitated from the blood filtrate by means of silver nitrate in the presence of nitric acid and the excess of silver titrated with standard thiocyanate solution, using ferric ammonium sulphate as an indicator.

Procedure.—Because of the slight variations in the chloride content of blood, dilution in preparation of protein-free filtrates should be made very carefully and volumetric flasks may be preferred.

Pipette 10 c.c. of the protein-free filtrate into a porcelain dish. Add with a pipette 5 c.c. of the standard silver nitrate solution[4] and stir thoroughly. Add

[1] Stander and Radelet, *Bull. Johns Hop. Hosp.*, 38, 423, 1926.

[2] Cori: *Jour. Biol. Chem.*, 63, 261, 1925.

[3] Whitehorn, J. C.: *Jour. Biol. Chem.*, 45, 449, 1921. This method is applicable to plasma and whole blood. The same principle was used by Rieger: *J. Lab. Clin. Med.*, 6, 44, 1920–21. Rappleye: *Jour. Biol. Chem.*, 35, 509, 1918, showed that the Volhard method could be applied directly to plasma without prior removal of protein. Van Slyke and Donleavy: *Jour. Biol. Chem.* 37, 551, 1919, showed that the iodometric method of McLean and Van Slyke: *Jour. Biol. Chem.*, 21, 361, 1915, could also be used in this way. Austin and Van Slyke: *Jour. Biol. Chem.*, 41, 345, 1920, used the picric acid precipitation in the case of whole blood. Myers and Short: *Jour. Biol. Chem.*, 44, 47, 1920, have combined the picric acid precipitation of protein with the Volhard titration to produce a convenient and satisfactory method. Wetmore; *Jour. Biol. Chem.*, 45, 113, 1920, uses copper hydroxide precipitation and the Volhard titration.

[4] Preparation of Reagents.—Dissolve 2.905 gm. of C.P. silver nitrate in distilled water. Transfer this solution to a liter volumetric flask and make up to the mark with distilled water. Mix thoroughly and preserve in a brown bottle. 1 c.c. = 1 mg. NaCl. (It is to be noted that the silver nitrate and nitric acid are not added to the protein-free filtrate simultaneously. To do so may result in the mechanical enclosure of silver nitrate solution within the curds, and a consequent error in the positive direction.)

Because sulfocyanates are hygroscopic, the standard solution should be prepared volumetrically. As an approximation about 1.7 gm. of KCNS or 1.4 gm. of NH_4CNS should be dissolved in a liter of water. By titration under the conditions specified under "Procedure" and by proper dilution prepare a standard such that 5 c.c. are equivalent to 5 c.c. of the silver nitrate solution.

The solid ferric alum is used rather than a solution, in order to insure a very high concentration in the mixture to be titrated. It is powdered in order to facilitate its solution.

about 5 c.c. of concentrated nitric acid (sp. gr. 1.42), mix, and let stand for 5 minutes, to permit the flocking out of the silver chloride. Then add with a spatula an abundant amount of powdered ferric ammonium sulfate (about 0.3 gm.) and titrate the excess of silver nitrate with the standard thiocyanate solution until the definite salmon-red (not yellow) color of the ferric thiocyanate persists in spite of stirring for at least 15 seconds.

Calculation.—[5.00(cc.$AgNO_3$ used) − x(cc.KCNS used)] 100 = mg. of NaCl per 100 c.c. of blood (or plasma).

Interpretation.—Whole blood normally contains from 450 to 500 mg. of sodium chloride per 100 c.c. and the plasma from 570 to 620 mg. Higher values are obtained in nephritis, and this determination may aid in deciding whether or not salt should be restricted in the diet. Increases in whole blood chlorides may also be observed in certain cardiac conditions, eclampsia, prostatic obstruction and anemia (due to the higher proportion of plasma). There may be a decrease of chloride in diabetes and fevers as well as in pneumonia with chloride retention. For accurate determinations of plasma chlorides the blood must be collected under oil (p. 434) because there is a shift of chlorides from cells to plasma when CO_2 diffuses out of the plasma into the air.

17A. Determination of Chlorides in Blood and Tissue (Van Slyke).[1] **Principle.** The proteins are destroyed, and the chloride is precipitated, by heating with concentrated nitric acid in the presence of known amounts of silver nitrate, and the excess silver is titrated with thiocyanate as described in Whitehorn's method. This procedure is applicable to tissues as well as blood.

Procedure.—One c.c. of serum or blood is placed in a 100 c.c. pyrex test tube and 3 c.c. of 0.05 N silver nitrate prepared in concentrated nitric acid[2] are added. The tube is covered with a watch glass and the mixture heated on a water bath till the solution above the AgCl precipitate is clear and light yellow in color (1–2 hours for serum, overnight for blood or tissue). 6 c.c. of a 5 per cent solution of ferric alum in water are added, the solution is cooled, and the excess silver is titrated with 0.02 N thiocyanate.[3] An empirical correction of 0.04 c.c. is subtracted from the thiocyanate used, as this excess is necessary to give a decided end-point.[4]

For tissues the procedure is the same except that digestion with 0.02 N $AgNO_3$ in nitric acid is advised instead of 0.05 N. It is also desirable to add a drop or two of caprylic alcohol to prevent foaming at the beginning of the digestion.

Calculation: $\frac{117\,(7.50 - \text{c.c. 0.02 N thiocyanate})}{\text{c.c. or gm. of sample}}$ = mg. NaCl per 100 c.c. or gm. Control titrations are made in which 3 c.c. of the 0.05 N $AgNO_3$ in HNO_3 plus 6 c.c. of ferric alum solution are titrated directly with 0.02 N thiocyanate. If the latter varies from 7.50 c.c., the thiocyanate figure in the formula is to be multiplied by $\frac{7.50}{\text{c.c. thiocyanate used in control}}$.

[1] Van Slyke: *Jour. Biol. Chem.*, 58, 523, 1923.

[2] Dissolve 8.495 gm. of fused $AgNO_3$ in a minimum amount of water and make up to one liter with nitric acid (sp. gr. 1.4).

[3] The thiocyanate is standardized against the silver solution as described in the Whitehorn procedure. The standardization should be repeated at least every two weeks.

[4] The original paper describes a macro method which is recommended for more accurate work.

18. Determination of Inorganic Phosphate. Method of Fiske and Subbarow.[1] **Principle.**—Phosphates react with ammonium molybdate to form ammonium phosphomolybdate. The latter is reduced by aminonaphtholsulphonic acid to a blue compound which is estimated colorimetrically.

Procedure.—Transfer to an Erlenmeyer flask 4 volumes of 10 per cent trichloroacetic acid.[2] **While the flask is being gently rotated, run in 1 volume of**

[1] Fiske and Subbarow: *J. Biol. Chem.*, 66, 375, 1925. This is a modification of the methods of Bell and Doisy, *J. Biol. Chem.*, 44, 55, 1920 and of Briggs, *J. Biol. Chem.*, 53, 13, 1922 and 59, 255, 1924.

[2] 10 *Per cent Trichloroacetic Acid.*—The quality of this reagent is of great importance. Certain impurities may markedly retard color development. Merck's U.S.P. product is free from any such contamination, but contains a trace of phosphate. The reagent should either be purified by distillation, or the amount of phosphate determined in each sample as follows: Arrange three tall beakers of 150 c.c. capacity on a piece of white paper. Into one of these (A) put 100 c.c. of water. In a second beaker (B) mix 85 c.c. of water, 10 c.c. of Molybdate I, and 4 c.c. of 0.25 per cent aminonaphtholsulphonic acid; the result should be a solution practically as colorless as water, without a trace of blue (otherwise one or more of the reagents already added contains phosphate). To the third beaker (C) add 40 c.c. of the trichloroacetic acid solution, 45 c.c. of water, 10 c.c. of Molybdate II, and 4 c.c. of the sulphonic acid reagent, stirring thoroughly with a clean glass rod. Into B now run 1 c.c. of a dilute phosphate solution containing 0.005 mg. of phosphorus per c.c., and mix well. Proceed in the same way, adding 1 c.c. of this phosphate solution at intervals of not less than 2 minutes, until B and C appear to have the same color when examined from above. The volume of phosphate solution which must be added to bring this about, multiplied by 0.05, is the correction (in mg. per 100 c.c.) to be subtracted from the result in the analysis of blood.

Molybdate I.—2.5 per cent ammonium molybdate in 5 N sulphuric acid. Dissolve 25 gm. of the salt in 200 c.c. of water. Rinse into a liter volumetric flask containing 500 c.c. of 10 N sulphuric acid. Dilute to the mark with water and mix.

Molybdate II.—2.5 per cent ammonium molybdate in 3 N sulphuric acid. Prepared as above, but with only 300 c.c. of 10 N sulphuric acid. (To be used only with blood filtrates in the determination of inorganic phosphate.)

0.25 *Per cent Aminonaphtholsulphonic Acid.*—Dissolve 0.5 gm. of the dry powder in 195 c.c. of 15 per cent sodium bisulphite, add 5 c.c. of 20 per cent sodium sulphite, stopper, and shake until dissolved. If the bisulphite solution is old, more than 5 c.c. of sulphite will be needed—in that event add more sulphite 1 c.c. at a time, shaking after each addition, until solution is complete. This reagent can be prepared in a few minutes (the powder need not be very accurately weighed), and if not left exposed to the air it should keep about 2 weeks. The solution is more stable the higher its acidity, hence no more sulphite should be added than is needed to dissolve the reducing agent.

1, 2, 4-*Aminonaphtholsulphonic Acid.*—This may be prepared from β-naphthol according to Folin's directions (*J. Biol. Chem.*, 51, 386, 1922) with a single alteration. The final product, after washing with cold water, still contains some colored material. To remove this, the crystals on the filter, while still wet, should be further washed with alcohol as long as any color is extracted.

The reagent may also be obtained in satisfactory condition by one recrystallization of "technical" aminonaphtholsulphonic acid (Eastman Kodak Co.,), as follows. Heat 1000 c.c. of water to about 90°, and dissolve in it 150 gm. of sodium bisulphite and 10 gm. of crystalline sodium sulphite. To this mixture add 15 gm. of the crude sulphonic acid, and shake until all but the amorphous impurity has dissolved. Filter the hot solution, through a large paper (about 32 cm.), cool the filtrate thoroughly under the tap, and add to it 10 c.c. concentrated hydrochloric acid. Filter with suction, wash with about 300 c.c. of water, and finally with alcohol until the washings are colorless.

The purified sulphonic acid should be dried in air with the least possible exposure to light, then powdered and transferred to a brown bottle.

Standard Phosphate (5 *c.c.* = 0.4 *mg. P*).—Dissolve 0.3509 gm. of pure monopotassium phosphate in water. Transfer quantitatively to a liter volumetric flask, add 10 c.c. of 10 N sulphuric acid, dilute to the mark, and mix. The standard keeps indefinitely.

15 *per cent Sodium Bisulphite.*—The solution must be free from turbidity before it can be used. Freshly prepared sodium bisulphite solutions may not filter clear, in which case 2 or 3 days standing (before filtering) will be necessary. Keep well stoppered.

20 *per cent Sodium Sulphite.*—Because it is likely to be of better quality, it is preferable

blood, (oxalate is the most suitable anticoagulant. Use 2, or at the most 3, mg. of potassium oxalate per c.c. of blood) plasma, or serum—as the case may be—from a pipette calibrated for delivery (not contents). Close the mouth of the flask with a clean dry rubber stopper, and shake vigorously a few times. Filter through an ashless paper (the mixture may be filtered at once).

Measure 5 c.c. of the filtrate into a tube graduated at 10 c.c. or a 10 c.c. volumetric flask. Add 1 c.c. of 2.5 per cent ammonium molybdate in 3 N sulphuric acid (Molybdate II), and finally (after mixing) 0.4 c.c. of the usual sulphonic acid reagent. Dilute to the mark and mix. The standard, to be prepared as nearly as possible at the same time, is identical with the standard used for urine (0.4 mg. of phosphorus in a volume of 100 c.c., or 0.2 mg. in a 50 c.c. flask with half as much of each reagent), so blood and urine may be read against the same solution. It should be noted that the molybdate reagent added to the standard is always the one containing 5 N sulphuric acid (Molybdate I), and is different from that used for the blood filtrate. The purpose of this is to compensate for the high concentration of trichloroacetic acid in the filtrate.

The reading, as with urine, may be made in about 5 minutes, but it should be repeated a few minutes later if the color is particularly strong.

Calculation.—To obtain the result in mg. of phosphorus per 100 c.c. of blood or serum, set the unknown at 20 mm., and multiply the reading of the standard by 0.2. From the figure so obtained subtract the correction for any phosphate which the trichloroacetic acid may contain. If the serum contains less than 2 mg. per cent of P, 1 c.c. of regular standard diluted 5 times (0.016 mg.) may be added to the filtrate before adding reagents or within 5 minutes thereafter.

Interpretation.—The normal inorganic phosphorus of the blood serum is about 5 mg. per 100 c.c. in infants and children and 3.7 mg. in the adult. In severe nephritis inorganic phosphorus may go up to 15–20 mg. and may bear a relation to the acidosis found in such cases. Clinically the most important changes are those in rickets. In the rickets of children or in experimental rickets produced in animals by low phosphorus diets the inorganic phosphorus of the blood may fall to 2 mg. or lower. Treatment with antirachitic vitamine (cod liver oil) or ultraviolet radiation increases the phosphorus of the blood and leads to increased calcification of the bones. During the healing period following fracture of a bone an increase in plasma phosphates is sometimes observed. The phosphate level of the plasma of children rises during the summer and falls during the winter. Rickets has its greatest incidence during the late winter, reaching its peak in March. These findings may be correlated with the degree of exposure to solar ultraviolet rays. Injections of insulin decrease the phosphate of the plasma. Phosphate is known to be concerned in carbohydrate metabolism.

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to use the crystalline sulphite [$Na_2SO_3\ 7H_2O$]. Dissolve 200 gm. of this in 380 c.c. of water. Remove any suspended matter by filtration, and keep stoppered.

10 *N Sulphuric Acid.*—450 c.c. of concentrated sulphuric acid added to 1300 c.c. of water.

Greenwald[1] has isolated diphospho-l-glyceric acid from the blood. From 30–40 per cent of the total acid soluble and 36–55 per cent of the organic acid soluble P of pig blood was found in this form. The substance may exist in the blood as an ester. Most of the lecithin of the blood is in the red cells which also contain most of the inorganic phosphate.[2]

18A. Determination of Total Acid-soluble Phosphorus in Blood. Method of Fiske and Subbarow. Principle.—Organic matter is destroyed by heating with nitric and sulphuric acids and phosphate determined colorimetrically as in the preceding method.

Procedure.—5 c.c. of the trichloroacetic acid filtrate should be used if possible. Boil this down, over a micro burner, in a large lipped Pyrex test-tube (200 by 25 mm.) with 5 c.c. of 5 N sulphuric acid (or 2.5 c.c. of 10 N) and a piece of quartz to prevent bumping. The bottom of the tube should be about 2 cm. above the burner tip. As soon as charring can be seen, or fumes appear, turn the flame down very low, so that the mixture barely boils, and heat until there is no further blackening. Now add 1 drop of nitric acid so that it runs down the wall of the test-tube—it should not fall directly into the digestion mixture. If the color does not promptly disappear, add another drop of nitric acid in the same manner, and continue in this way until there is no color left. Nothing is gained by using a large amount of nitric acid. Ordinarily a single drop will be enough, and then about 30 seconds further boiling with the same low flame (to remove most of the nitric and nitrous acids left) will complete the ashing process.

Cool the tube under the tap, rinse the contents into a 50 c.c. volumetric flask with 35 c.c. of water, add 5 c.c. of Molybdate III[3] and 2 c.c. of the reducing agent (0.25 per cent aminonaphtholsulphonic acid solution). Dilute to the mark, and proceed as usual, reading against the standard that has been described before.[4]

Calculation.—By setting the unknown at 20 mm., the reading of the standard will give directly the total acid-soluble phosphorus in mg. per 100 c.c. of blood.

18B. Determination of Phosphorus in Tissues and Other Biological Material.—The colorimetric method described above may be used for tissues and other biological material. It is nearly always necessary first to ash. Urine, blood, lipoid extracts, etc. may be treated according to directions given for blood filtrates except that more nitric acid will naturally be needed. Such applications should be checked by dry ashing methods to make sure that phosphorus is not being lost. Dry

[1] Greenwald: *Jour. Biol. Chem.*, 63, 339, 1925.

[2] For determination of lipoid phosphorus of the blood see p. 395.

[3] Molybdate III—2.5 per cent ammonium molybdate in water. As soon as any considerable amount of sediment (ammonium trimolybdate) has appeared in this solution, it should be discarded.

[4] The analysis may be made with 1 c.c. of filtrate, using 1 c.c. of 5 N sulphuric acid. The procedure is then otherwise the same as that described above, except that the final dilution must be 10 c.c. (instead of 50) and the reagents diminished in proportion. It is safer, in this case, to use a smaller test-tube (about 10 mm. in diameter) for the digestion, The most probable cause of loss of phosphate is superheating at the edge of the meniscus. which should consequently be as far removed as possible from the source of heat.

ashing is frequently the simplest method. If little organic matter is present ignition with a small amount of sodium carbonate will suffice. In other cases, as with urine, magnesium nitrate (1 c.c. of 10 per cent) is satisfactory. For tissues, carbonate-nitrate fusion mixtures are perhaps best. Porcelain dishes may generally be used but blanks should be run on them. Some silica will not interfere. If platinum is used dissolve most of the ash with water before adding acid to remove the last of the material as nitrous acid attacks platinum. Evaporate the total solution to dryness in a beaker or porcelain dish (covered with a watch glass as long as carbon dioxide is evolved). Dissolve residue in water, make to volume and determine as usual.

18C. Determination of Inorganic Phosphate. Method of Benedict and Theis.[1] **Principle.**—The principle is the same as that of the method of Fiske and Subbarow. A hydroquinone-sulphite solution is used as the reducing agent.

Procedure.—Measure 5 c.c. of the filtrate obtained as in the method of Fiske and Subbarow into a test tube. Add 3 c.c. of distilled water and 1 c.c. of special molybdate solution[2] diluted just before use with an equal volume of concentrated sulphuric acid. Add 1 c.c. of the hydroquinone-sulphite solution. Stopper, invert to mix, and put in boiling water bath for 10 minutes together with another tube containing 5 c.c. of standard phosphate solution (0.025 mg. P) and the reagents. Compare in a colorimeter.

Calculation.—With the unknown set at 12.5 mm., multiply the reading of the standard by 0.1 to get mg. P per 100 c.c. serum.

19. Determination of Inorganic Sulphate in Blood. Method of Denis.[3] **Principle.**—The sulphate is determined nephelometrically by the turbidity produced with barium chloride.

Procedure.—*For Animal Blood.* Use 30 mg. sodium citrate per 10 c.c. of blood to prevent coagulation. Treat 10 c.c. of this blood with 10 c.c. 0.02 N HCl and 5 minutes later 30 c.c. of 5 per cent mercuric chloride solution, containing 5 c.c. concentrated HCl per liter. Shake well, let stand 1 hour, and filter. To 10 c.c. of filtrate add 5 c.c. of 1 per cent ammonium nitrate solution and with stirring 5 c.c. of 1.0 per cent barium chloride containing 5 c.c. of concentrated HCl per liter. After 10 minutes compare with a standard (10 c.c. K_2SO_4 = 0.10 mg. S) to which have been added 10 c.c. of the $HgCl_2$, 10 c.c. of the NH_4NO_3 and 10 c.c. of the $BaCl_2$ solution.

For Human Blood.—Treat 5 c.c. of oxalated blood or plasma with 5 c.c. 0.1 N HCl, 5 c.c. 5 per cent $HgCl_2$ and 0.3 gm. finely powdered $HgCl_2$. Shake vigorously for 5 minutes and at intervals for 1 hour. Filter. Treat 5 c.c. of filtrate

[1] Benedict and Theis: *Jour. Biol. Chem.*, 61, 63, 1924.

[2] *Molybdate Solution.*—To 20 gm. molybdic acid (free from ammonia) add 25 c.c. 20 per cent NaOH. Warm gently to dissolve. Cool, dilute to 250 c.c. and filter. *Hydroquinone-sulphite solution.*—Dissolve 30 gm. sodium bisulphite in 100 c.c. water in a 200 c.c. volumetric flask. Add 1 gm. hydroquinone. Mix and make to volume. *Phosphate standard.* Dilute 5 c.c. stock solution (0.4394 gm. KH_2PO_4 per liter) to 100 c.c. with water, and add chloroform to preserve.

[3] Denis: *Jour. Biol. Chem.*, 49, 311, 1921.

with 1 c.c. NH_4NO_3 and 1 c.c. $BaCl_2$. After 10 minutes compare turbidity with a standard prepared from 10 c.c. of K_2SO_4 solution (= 0.1, 0.05, or 0.03 mg. S), 10 c.c. $HgCl_2$, 4 c.c. NH_4NO_3 and 4 c.c. $BaCl_2$. See nephelometric calculations, p. 365.

Interpretation.—Human blood contains from 0.5–1.0 mg. S, animal bloods from 2–3 mg. In a case of nephritis with 200 mg. non-protein N the values were 12–16 mg. S.

19A. Determination of Total Sulphate in Blood. Method of Denis and Leche.[1] **Principle.**—The blood is heated with acid in an autoclave to put the protein in solution and the sulphate is precipitated with barium.

Procedure.—To 25 c.c. of citrated blood add 55 c.c. of distilled water and 20 c.c. of 20 per cent trichloroacetic acid solution. Shake well. Let stand 1 hour and filter. To 25 c.c. of clear filtrate add 75 c.c. distilled water and 20 c.c. of 5 per cent barium chloride. Let stand over night. Transfer to a tared Gooch crucible with an asbestos mat, wash, dry, and ignite to constant weight. Normal blood contains from 1–2.5 mg. of sulphate S.

19B. Total Sulphate in Tissues. Procedure.—Place 10 gm. of tissue (finely divided by passing through a meat grinder) in a large (25 × 200 mm.) Pyrex test tube. Add 20 c.c. approximately N HCl. Close with a small watch glass held in place with a square of tin foil. Heat in an autoclave at 200°C. for 1 hour. An autoclave withstanding 60 atmospheres may be used. Or heat in bacteriological autoclave at 150°C. for 2 hours. Protein is decomposed. There may be some undecomposed fat on the surface. Transfer quantitatively to a 100 c.c. volumetric flask. Make to mark. Mix. Transfer to two 50 c.c. centrifuge tubes and centrifuge for 5 minutes. Filter by suction through Gooch crucible with asbestos mat. To 40 c.c. of the filtrate in a 250 c.c. beaker add 10 c.c. of 5 per cent barium chloride drop by drop from a burette. Heat to boiling, let stand over night and transfer to a tared Gooch crucible, wash, dry, and ignite to constant weight. Brain tissue may contain about 10 mg. sulphate S per 100 gm. and liver tissue as high as 20. Most other tissues give values of 1–5 mg. Somewhat variable results are obtained. There is little absorption of sulphates by tissues following injections.

19C. Determination of Total Sulphur in Biological Material. Method of Stockholm and Koch.[2] **Principle.**—The complete oxidation of biological material for the sulphur determination is often a difficult procedure. The following method in which strong hydrogen peroxide and nitric acid are used is believed to be the most effective.

Procedure.—Into a 100 c.c. nickel crucible (50 × 70 mm.) containing 10 c.c. of the 25 per cent sodium hydroxide solution is transferred 0.5 to 2.0 gm. of the substance. The covered crucible is then heated on the steam-bath until the mass is almost dry. This requires several hours, but causes considerable decomposition of the complex substances present, so that the sulphur in particular

[1] Denis and Leche: *Jour. Biol. Chem.*, 65, 561 and 565, 1925.
[2] Stockholm and Koch: *Jour. Am. Chem. Soc.* 45, 1953, 1923.

can later be easily oxidized. In case the evaporation has proceeded too rapidly it is best to add again 10 c.c. of water and to repeat the slow evaporation. To the slightly moist material 5 c.c. of 30 per cent H_2O_2[1] is added very gradually. In some cases it is necessary to stir the mass with a glass rod or to add a few drops of water so as to distribute the reagent properly. During this treatment the heating is continued on the steam-bath.

The material thus partially oxidized is next transferred to a 300 c.c. Kjeldahl flask, acidified with nitric acid and concentrated over a free flame until salts begin to separate. This concentrated solution is then oxidized, while boiling, by the gradual addition of fuming nitric acid and bromine until 10 c.c. of acid and 40 to 50 drops of bromine have been used. With material low in, or free from, fat this treatment is usually sufficient to bring about complete oxidation of the organic matter. The solution is next evaporated almost to dryness and after water has again been added evaporation is repeated to remove most of the nitric acid. When the water solution of this material is not absolutely clear it is filtered and after it has been neutralized with sodium hydroxide and diluted to about 600 c.c. it is acidified by the addition of 10 c.c. of conc. hydrochloric acid. 10 c.c. of 0.1 N sulfuric acid is added and after the mixture has been heated to boiling, 10 c.c. of a 10 per cent barium chloride solution is added, drop by drop. The boiling is continued for 10 to 15 minutes and then the mixture is heated for 10 to 12 hours before it is filtered and washed as usual. The filter paper and precipitate are cautiously burned and weighed. Blank estimations are made in exactly the same way.

20. Determination of Calcium in Serum. Clark-Collip Modification of the Kramer-Tisdall Method.[2] **Principle.**—Calcium is precipitated directly from the serum as oxalate and the latter is titrated with potassium permanganate.

Procedure.—Introduce into a graduated 15 c.c. centrifuge tube 2 c.c. of clear serum, 2 c.c. of distilled water, and 1 c.c. of 4 per cent ammonium oxalate solution. Mix thoroughly. The centrifuge tube should have an outside diameter of 6–7 mm. at the 0.1 c.c. mark. Mixing is aided by holding the tube at the mouth and giving it a circular motion by tapping the lower end. Let stand for 30 minutes or longer. Again mix the contents. Centrifuge for about 5 minutes at 1500 revolutions per minute. Carefully pour off the supernatant liquid and while the tube is still inverted let it drain in a rack for 5 minutes, resting the mouth of the tube on a pad of filter paper.[3] The mouth of the tube is wiped dry with a soft cloth. Stir up the precipitate and wash the sides of the tube with 3 c.c. of dilute ammonia (2 c.c. of concentrated ammonia to 98 c.c. of water) directed in a very fine stream, from a wash bottle. The suspension is centrifuged and drained again as before. Add 2 c.c. of approximately normal sulphuric acid (28 c.c. of concentrated acid to a liter) this being blown from a pipette directly upon the

[1] Sold commercially as Perhydrol or Superoxal.

[2] Clark and Collip: *Jour. Biol. Chem.*, 63, 461, 1925; Tisdall: *Jour. Biol. Chem.*, 56, 439, 1923; Kramer and Tisdall: *Jour. Biol. Chem.*, 47, 475, 1921; Clark: *Jour. Biol. Chem.*, 49, 487, 1921. A method for the titration of serum Ca as $CaCO_3$ has been described by Trevan and Bainbridge: *Bioch. Jour.*, 20, 423, 1923.

[3] To insure uniform drainage the tubes should always be cleaned thoroughly by heating at approximately 100° for a few minutes in a cleaning mixture made by adding 1500 c.c. of concentrated sulphuric acid to a solution of 200 gm. of sodium dichromate in 100 c.c. of water.

precipitate so as to break up the mat and facilitate solution. Place tube in a boiling water bath for about 1 minute. Titrate with 0.01 normal potassium permanganate[1] to a definite pink color which persists for at least one minute. If necessary during the course of the titration warm the tube by placing in a water bath kept at 70–75°. A microburette graduated in 0.02 c.c. should be used.

Calculation.—One c.c. of 0.01 N $KMnO_4$ is equivalent to 0.2 mg. Ca. The number of mg. of Ca per 100 c.c. of serum therefore equals $\frac{100 \times 0.2 \times x}{2} = 10x$, where x equals the number of c.c. of permanganate required. From x subtract the blank (number of c.c. of permanganate required to titrate 2 c.c. of the sulphuric acid solution to the usual end point) before making the calculation.

Interpretation.—Normal human blood serum contains from 9–11 mg. of calcium per 100 c.c., the values being slightly higher for children than for the adult. After parathyroidectomy the blood calcium falls to a low level and the injection of parathyroid extract results in a considerable increase. In infantile tetany values of 3.5 to 7.0 mg. have been observed. In severe nephritis the calcium content may decrease to 7.0 mg. or less. A considerable part of the calcium is non-diffusible.[2]

20A. Determination of Calcium in Tissues and Other Biological Materials. Method of Corley and Denis.[3] Where the amount of organic matter to be destroyed is small the material may be ashed in silica or platinum crucibles with the aid of a few drops of nitric acid to destroy the last carbon. Feces, milk, and bone may usually be handled in this way. Tissues low in calcium present a more difficult problem and Corley and Denis have suggested autoclaving such materials with alkali.

10 gm. of finely ground tissue together with 50 c.c. of 0.1 N sodium hydroxide are placed in a large Pyrex test-tube (25 × 200 mm.) graduated at 60 c.c. The tube is covered with tin-foil and autoclaved for 2 hours at a temperature of 180°C. by which treatment practically complete solution is effected. The solution is now rendered strongly acid by the addition of concentrated hydrochloric acid, diluted with distilled water to a volume of 60 c.c., allowed to stand for at least 30 minutes

[1] 0.01 N $KMnO_4$. (Halverson and Bergeim: *J. Ind. Eng. Chem.*, 10, 119, 1918). Dissolve 0.4 gm. pure potassium permanganate in one liter of redistilled water in a thoroughly clean Florence flask. Insert funnel covered with a watch glass as a condenser and digest for several hours at near the boiling point. Cool, let stand over night and filter with gentle suction through a 3 inch Büchner funnel lined with ignited asbestos. Transfer to a perfectly clean glass stoppered bottle and keep in a dark place. Permanganate solution prepared by dilution of 0.1 N solution must be standardized immediately before use a its deteriorates rapidly and comes to a constant value only after several days. The 0.01 N permanganate is standardized against 0.01 N sodium oxalate which should keep for several months. Dry highest purity sodium oxalate in an oven at 100–105° for 12 hours. Dissolve exactly 0.67 gm. of the oxalate in redistilled water, add 5 c.c. of conc. H_2SO_4 and dilute to one liter. Mix well. Transfer exactly 25 c.c. of this solution to a 100 c.c. Erlenmeyer flask, add 1 c.c. conc. H_2SO_4, warm to about 70° and titrate with the permanganate solution. The permanganate solution should be frequently restandardized although after the first few days if carefully prepared and kept it should not deteriorate more than 0.1 per cent per week.

[2] For method of determining diffusible calcium see Moritz: *Jour. Biol. Chem.*, 64, 81, 1925.

[3] Corley and Denis: *Jour. Biol. Chem.*, 66, 601, 1925.

and then filtered by suction through a Gooch crucible provided with a mat of calcium-free asbestos fiber or filter paper. Centrifugation greatly facilitates the latter operation.

At this stage the solution should be clear and absolutely free from suspended particles; the color, however, varies greatly from the light yellow given by muscle to the dark reddish brown noted when liver is used. An aliquot of the solution (usually 15 c.c. in the case of normal tissues) is measured into a 50 c.c. conical centrifuge tube, a half volume of 4 per cent ammonium oxalate solution is added, and the reaction is made alkaline to methyl red by the cautious addition of concentrated ammonium hydroxide. This precipitation should be made shortly after filtration, as if the filtrate is allowed to stand too long the color gradually deepens and the further procedure becomes more difficult. After standing for at least 1 hour the precipitate is centrifuged down, and the supernatant liquid is poured off as completely as possible without disturbing the precipitate.

This precipitate is now dissolved in 5 c.c. of approximately normal sulphuric acid and titrated at room temperature with 0.1 N potassium permanganate until pink, and after a period of 5 to 10 minutes there are added 5 c.c. of a 4 per cent solution of ammonium oxalate. As soon as the solution is perfectly clear 5 c.c. of water are added, and the reaction is adjusted to the neutral point of methyl red by the cautious addition of concentrated ammonium hydroxide, using approximately normal hydrochloric acid for back titrations if the reaction has passed the neutral point.

After standing for 1 hour or longer if convenient the precipitate is centrifuged down, the supernatant liquid poured off as completely as possible, and the calcium oxalate washed with 30 to 35 c.c. of ice-cold water, and centrifuged again. After decantation of the wash water, the precipitate is dissolved in 5 c.c. of approximately normal sulphuric acid, the tube placed for 5 minutes in a water bath at 70°C., and the solution then titrated with 0.01 N potassium permanganate.

Calculation.—The blank titration given by the sulphuric acid is subtracted. Since 1 c.c. of 0.01 N potassium permanganate is equivalent to 0.2 mg. of calcium, the calculation is as follows:

$$\text{mg. Ca per 100 gm. sample} = (\text{titration} - \text{blank}) \times \frac{0.2 \times 60}{\text{aliquot}} \times \frac{100}{\text{sample in gm.}}$$

Using a 10 gm. sample of tissue and a 15 c.c. aliquot this equals the titration minus the blank, times 8.

21. Determination of Magnesium in Serum. Method of Denis.[1] **Principle.**—After removal of calcium as oxalate the magnesium is precipitated as magnesium ammonium phosphate and the latter is estimated by a colorimetric phosphate determination.

Procedure.—Precipitate calcium from 2 c.c. of serum as in the procedure for calcium in serum (see p. 408). After centrifugation pipette 3 c.c. of the supernatant fluid into a 15 c.c. centrifuge tube and add with stirring 0.5 c.c. of 5 per cent ammonium phosphate solution containing 5 c.c. of concentrated ammonium hydroxide solution per liter. Let stand over night; centrifuge, siphon off the supernatant fluid and wash the tube with 5 c.c. of a mixture of 1 part concentrated ammonium hydroxide (sp. gr. 0.9) and 2 parts of water. Centrifuge and siphon

[1] Denis: *Jour. Biol. Chem.*, 52, 411, 1922.

off wash liquid. Repeat this washing a second and third time and then wash finally with 5 c.c. of 75 per cent alcohol containing 10 c.c. of concentrated ammonium hydroxide per liter. Siphon off again and let stand in a warm place until the ammonia has evaporated.

Dissolve the precipitate of magnesium ammonium phosphate in 5 c.c. of 0.1 N hydrochloric acid and transfer with the aid of 5 c.c. more of the acid to a 25 c.c. volumetric flask. As a standard introduce into another 25 c.c. flask 10 c.c. of a solution of magnesium ammonium phosphate (containing 0.02 mg. magnesium in 10 c.c.[1]) in 0.1 N hydrochloric acid. To each flask add 2 c.c. of 2.5 per cent solution of ammonium molybdate in water and 1 c.c. of 0.25 per cent aminonaphtholsulphonic acid solution (see phosphate method p. 403). Diluted to mark, mix, let stand for 5 minutes. Compare in a colorimeter.

Calculation.—If 3 c.c. of the 5 c.c. of supernatant fluid obtained from 2 c.c. of serum be used this will correspond to 1.2 c.c. of serum. As the standard contains phosphate equivalent to 0.02 mg. magnesium,

$$\text{mg. of Mg per 100 c.c. of serum} = \frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.02 \times \frac{100}{1.2}$$

Interpretation.—Normally about 2 to 3 mg. of mg. are found in 100 c.c. of serum and about 1.6 mg. in 100 c.c. of blood. No characteristic changes have been observed in pathological conditions.

22. Determination of Sodium in Serum. Method of Kramer and Gittleman.[2] **Principle.**—Sodium is precipitated as the pyroantimonate

$$K_2H_2Sb_2O_7 + 2NaCl \rightarrow Na_2H_2Sb_2O_7 + 2KCl$$

and the antimony in the precipitate titrated iodometrically.

Procedure.—Place 2 c.c. of serum (or the ash of an equal amount of serum dissolved in 2 c.c. 0.1 N hydrochloric acid and made alkaline with 4 drops of 1.8 N alcohol-washed potassium hydroxide) in a 50 c.c. tapering graduated Pyrex centrifuge tube preferably coated with a thin layer of paraffin. Add 10 c.c. of the pyroantimonate reagent[3] and exactly 3 c.c. of 95 per cent alcohol (redistilled over KOH) drop by drop while stirring with a rubber tipped glass rod. Stopper with a cork and let stand for 30 minutes. Then centrifuge for 5 minutes.

[1] A solution of 0.202 gm. $MgNH_4PO_4.6H_2O$ in 100 c.c. 0.1 N HCl is diluted exactly 1:10 with 0.1 N HCl.

[2] Kramer and Gittleman: *Jour. Biol. Chem.*, 62, 353, 1924. See Note 3, p. 412.

[3] *Potassium Pyroantimonate Reagent.*—500 c.c. of distilled water are heated to boiling in a Pyrex flask and approximately 10 gm. of potassium pyroantimonate (J. T. Baker) are added. The boiling is continued from 3 to 5 minutes, the flask immediately cooled under running water, and when the contents are cold 15 c.c. of 10 per cent KOH (alcohol-washed) are added. The reagent is filtered through ash-free filter paper into a paraffined bottle. Frequently some of the undissolved potassium pyroantimonate will pass through even the best filter paper. If the reagent is allowed to stand 24 hours after filtering, all the undissolved potassium pyroantimonate will settle to the bottom. The supernatant fluid is then clear and may be used as long as it remains so. The reagent keeps perfectly well at room temperature for at least 1 month. 10 c.c. of this reagent will precipitate 11 mg. of sodium. The 10 per cent KOH should also be kept in a paraffined bottle.

Before the reagent is used for the first time, it should be tested for the presence of sodium. It should also be ascertained that none of the potassium pyroantimonate is precipitated by the addition of alcohol in the proportion used in the method. This is accomplished by adding to 10 c.c. of the reagent in a platinum dish 2 c.c. of distilled water and 3 c.c. of 95 per cent alcohol.

Siphon off all but 2 c.c. of supernatant fluid. Add 10 c.c. 30 per cent alcohol mix with supernatant fluid and again centrifuge. All supernatant fluid possible is then removed with a suitable pipette and rubber bulb.

Add to the precipitate 5 c.c. 10 N hydrochloric acid (concentrated acid sp. gr. 1.182) and aid solution by stirring thoroughly with a glass rod. Transfer to a 250 c.c. Pyrex beaker tall form, completing transfer with not more than 10 c.c. of distilled water. Stir until solution is complete. Add 2 c.c. of 20 per cent potassium iodide solution and titrate at once with 0.1 N sodium thiosulphate. A 15 c.c. burette graduated in 0.02 c.c. is convenient. Add the thiosulphate very rapidly with constant stirring until the brown color is practically gone. Add 0.5 c.c. of 1 per cent freshly prepared soluble starch solution. Titrate as before until the sample turns brown, then very slowly with thorough stirring between additions. The end point is reached when the solution becomes water clear.

Calculation.—One equivalent of iodine is freed by the amount of antimony bound to 0.5 equivalent of Na. Therefore 1 c.c. N thiosulphate equals $\frac{2.3}{2}$ = 1.15 mg. Na. Hence if 2 c.c. of serum are used the number of mg. of Na per 100 c.c. of serum or solution = (c.c. thiosulphate used) × (thiosulphate factor) × $1.15 \times \frac{100}{2}$.

Interpretation.—There are normally found about 330 mg. of Na per 100 c.c. of plasma or serum, and about 200 mg. per 100 c.c. of whole blood. There is very little sodium in the corpuscles. Even in advanced nephritis the amount of sodium and the ratio of sodium to potassium in the plasma may not be significantly altered.[1] Sodium plays an important part in the acid base balance of the blood.

23. Determination of Potassium in Serum.[2] Method of Kramer and Tisdall.[3] Principle.—The potassium is precipitated as cobaltinitrite and the latter titrated with potassium permanganate.

Procedure.—Pipette 1 c.c. of serum into a 15 c.c. graduated centrifuge tube previously cleaned with bichromate sulphuric acid mixture and rinsed with water. Add drop by drop with agitation 2 c.c. of the sodium cobaltinitrite reagent.[4]

[1] Rabinowitch: *Jour. Biol. Chem.*, 62, 668, 1926.

[2] Doisy and Bell (Briggs: *Jour. Biol. Chem.*, 57, 351, 1923) have developed a colorimetric method for potassium based on diazotization with the nitrite group of potassium cobaltinitrite. Fiske and Litarczek (*Jour. Biol. Chem.*, 67, xvii, 1926) ash the cobaltinitrite precipitate, take up the potassium with a small volume of water and precipitate with alcoholic tartaric acid. The acid potassium tartrate is filtered off, washed with alcohol and titrated with 0.02 N sodium hydroxide.

[3] Kramer and Tisdall: *Jour. Biol. Chem.*, 46, 339, 1921. Kerr (*Jour. Biol. Chem.*, 67, 689, 1926) believes that more reliable results are obtained by performing the sodium and potassium determinations on the protein-free filtrate obtained after precipitation with trichloroacetic acid. The latter is subsequently decomposed by evaporation and treatment with concentrated HNO_3.

[4] Reagents required.

1. *Sodium Cobaltinitrite Reagent.* Solution A.—25 gm. of cobalt nitrate crystals (J. T. Baker) are dissolved in 50 c.c. of water and to this solution are added 12.5 c.c. of glacial acetic acid.

Solution B.—120 gm. of sodium nitrite (potassium-free) (Merck) are dissolved in 180 c.c. of water. This gives a total volume of about 220 c.c. To all of Solution A are added 210 c.c. of Solution B. An evolution of nitric oxide gas occurs at once. Air is drawn

Ash solutions are made up to 2 c.c. and treated with 1 c.c. of reagent. After 45 minutes add 2 c.c. of water, mix and centrifuge for 30 minutes at 1300–1400 revolutions per minute. By means of a siphon tube, the tip of which is turned upward, draw off all but 0.3 c.c. of fluid. Do not disturb the precipitate. Run 5 c.c. of water down the side of the tube. Gently agitate so that water is well mixed with residual reagent, the precipitate being disturbed as little as possible. To do this hold tube vertically and strike lower end gently with a circular motion. Centrifuge for 5 minutes. Repeat washing three times more. The final supernatant fluid should be clear. After siphoning this off add an excess of 0.02 N potassium permanganate (usually 2 c.c.) and 1 c.c. of approximately 4 N sulphuric acid. Mix precipitate well with fluid by means of a glass rod. Heat in a boiling water bath until no further change in color occurs (from 1–1½ minutes). Add enough 0.01 N sodium oxalate to decolorize solution completely (usually 2 c.c.). Titrate excess of oxalate with 0.02 N permanganate.

Calculation.—One c.c. 0.01 N permanganate = 0.071 mg. potassium. Therefore if 2 c.c. of permanganate are originally added and 0.43 c.c. more used in the final titration and 2 c.c. of 0.01 N oxalate are used the number of mg. of potassium in 100 c.c. of serum = [2.43 c.c. − 0.03 (the blank)] × 2 (to convert 0.02 N to 0.01 N) − 2 (c.c. 0.01 N oxalate) × 0.071 × 100 = 19.9 mg.

Interpretation.—The potassium concentration of normal human blood serum is relatively constant, ranging from 16–22 mg. per 100 c.c. Pathologically increased values have been reported in uremia and possibly eclampsia, though data are too limited to permit any definite conclusions. The corpuscles contain a high concentration of potassium, so that in primary or secondary anemia the values for whole blood, which are normally between 150 and 250 mg. per 100 c.c., may be low.

24. Deterimination of Iron in Foods and Other Biological Material.—Method of Walker.[1]**—Principle.**—Colorimetric determinations of iron as thiocyanate are convenient and delicate. The material is ashed. If only small amounts of phosphate are present the ash may be dissolved in hydrochloric acid (method of Thomson[2]). See p. 788. If moderate amounts of phosphate are present (not over 0.05 gm. phosphoric acid) the method of Walker using nitric acid is recommended. If large amounts of phosphate are present as compared with iron (as in milk) the method of Elvehjem and Hart[3] should be used.

through the solution until all the gas has passed off. The reagent (thus prepared has a pH of 5.7) is placed in the ice chest and filtered each time before using. It will keep at least 1 month.

2. *Approximately 4 N Sulphuric Acid.*—20 c.c. of concentrated sulphuric acid are poured into distilled water and diluted to 100 c.c.

3. 0.02 *N Potassium Permanganate.*—This is prepared by diluting N or 0.1 N potassium permanganate and is standardized after each series of determinations against the 0.01 N sodium oxalate (Sørensen).

4. 0.01 *N Sodium Oxalate.*—This is prepared from 0.1 N sodium oxalate. Thelatter is made by dissolving 6.7 gm. of Sørensen's sodium oxalate in a liter of water with the aid of 5 c.c. of concentrated sulphuric acid.

[1] Walker: *Analyst*, 50, 1279, 1925.

[2] Thomson: *J. Chem. Soc.*, 47, 493, 1885.

[3] Elvehjem and Hart: *Jour. Biol. Chem.*, 67, 43, 1926.

Procedure.—Weigh a 5 gm. sample into a porcelain evaporating dish. Ignite in an electric furnace. After cooling add 10 c.c. of distilled water and 3 c.c. of concentrated nitric acid. Filter off the insoluble residue. Treat the filtrate with 2 c.c. of hydrogen peroxide solution. After standing for 1 minute add 5 c.c. of 10 per cent potassium thiocyanate. Dilute to 50 c.c. Compare with 1 c.c. of a standard iron solution,[1] treated in the same way except that 2 c.c. of concentrated nitric acid are used. The extra 1 c.c. of acid added to the ash is neutralized by the alkalinity of the latter.

25. Determination of Iodine in Blood and Tissues. (See Chapter XX, p. 483).

OTHER METHODS OF BLOOD ANALYSIS

Methods for the analysis of blood for alkali reserve, gases, hemoglobin and for hydrogen ion concentration are given in the chapter on "Respiratory Metabolism and Neutrality Regulation," p. 419.

SPECTROSCOPIC EXAMINATION OF BLOOD[2]

Either the *direct*-vision spectroscope (Fig. 124) or the *angular*-vision spectroscope (Figs. 125 and 126) may be used in making the spectroscopic examination of the blood. For a complete description of these instruments the student is referred to any standard text-book of physics.

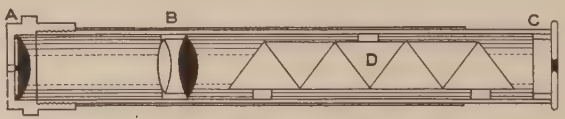


FIG. 124.—DIRECT-VISION SPECTROSCOPE.

1. Oxyhemoglobin.—Examine dilute (1:50) defibrinated blood spectroscopically. Note the broad absorption band between D and E. Continue the dilution until this single broad band gives place to two narrow bands, the one nearer the D line being the narrower. These are the typical absorption bands of oxyhemoglobin obtained from dilute solutions of blood. Now dilute the blood very freely and note that the bands gradually become more narrow and, if the dilution is sufficiently great, they finally entirely disappear.

2. Hemoglobin (so-called Reduced Hemoglobin).—To blood which has been diluted sufficiently to show well-defined oxyhemoglobin absorption bands add a small amount of Stokes' reagent.[3] The blood immediately changes in color from a bright red to violet-red. The oxyhemoglobin has been reduced through the action of Stokes' reagent and hemoglobin (so-called reduced hemoglobin) has been formed. This has been brought about by the removal of some of the loosely

[1] *Standard Iron Solution.*—Dissolve 0.7 gm. ferrous ammonium sulphate (dried to constant weight) in 100 c.c. distilled water. Add 5 c.c. conc. sulphuric acid. Warm slightly and add $KMnO_4$ solution until oxidation is complete. Dilute to 1 liter. One c.c. = 0.1 mg. Fe.

[2] For absorption spectra see plates I and II.

[3] Stokes' reagent is a solution containing 2 per cent ferrous sulphate and 3 per cent tartaric acid. When needed for use a small amount should be placed in a test-tube and ammonium hydroxide added until the precipitate which forms on the first addition of the hydroxide has entirely dissolved. This produces *ammonium ferrotartrate* which is a reducing agent.

combined oxygen from the oxyhemoglobin. Examine this hemoglobin spectroscopically. Note that in place of the two absorption bands of oxyhemoglobin we now have a single broad band lying almost entirely between D and E. This is the typical spectrum of hemoglobin. If the solution showing this spectrum be

FIG. 125.—ANGULAR-VISION SPECTROMETER.

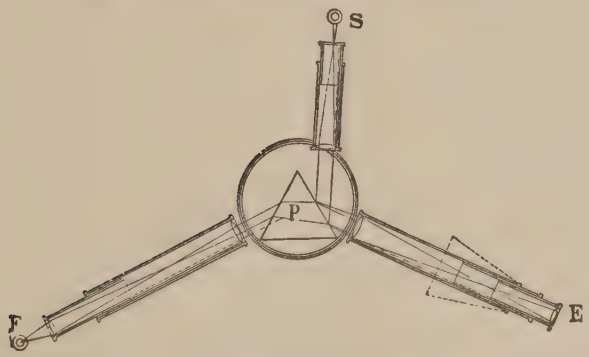


FIG. 126.—DIAGRAM OF ANGULAR-VISION SPECTROSCOPE. (*Long.*)

The white light *F* enters the collimator tube through a narrow slit and passes to the prism, *P*, which has the power of refracting and dispersing the light. The rays then pass to the double convex lens of the ocular tube and are deflected to the eyepiece *E*. The dotted lines show the magnified virtual image which is formed. The third tube contains a scale whose image is reflected into the ocular and shown with the spectrum. Between the light *F* and the collimator slit is placed a cell to hold the solution undergoing examination.

shaken in the air for a few moments it will again assume the bright red color of oxyhemoglobin and show the characteristic spectrum of that pigment.

3. **Carbon Monoxide Hemoglobin.**—The preparation of this pigment may be easily accomplished by passing ordinary illuminating gas[1] through defibrinated

[1] The so-called water gas with which ordinary illuminating gas is diluted contains usually as much as 20 per cent of carbon monoxide (CO).

ox-blood. Blood thus treated assumes a brighter tint (carmine) than that imparted by oxyhemoglobin. In very dilute solution oxyhemoglobin appears yellowish red whereas carbon monoxide hemoglobin under the same conditions appears bluish red. Examine the carbon monoxide hemoglobin solution spectroscopically. Observe that the spectrum of this body resembles the spectrum of oxyhemoglobin in showing two absorption bands between D and E. The bands of carbon monoxide hemoglobin, however, are somewhat nearer the violet end of the spectrum. Add some Stokes' reagent to the solution and again examine spectroscopically. Note that the position and intensity of the absorption bands remain unaltered.

The following are delicate chemical tests[1] for the detection of carbon monoxide hemoglobin:

Tannin Test.—Divide the blood to be tested into two portions and dilute each with 4 volumes of distilled water. Place the diluted blood mixtures in two small flasks or large test-tubes and add 20 drops of a 10 per cent solution of potassium ferricyanide.[2] Allow both solutions to stand for a few minutes, then stopper the vessels and shake one vigorously for 10–15 minutes, occasionally removing the stopper to permit air to enter the vessel.[3] Add 5–10 drops of ammonium sulphide (yellow) and 10 c.c. of a 10 per cent solution of tannin to each flask. The contents of the shaken flask will soon exhibit the formation of a dirty olive-green precipitate, whereas the flask which was not shaken and which, therefore, still contains carbon monoxide hemoglobin, will exhibit a bright red precipitate, characteristic of carbon monoxide hemoglobin. This test is more delicate than the spectroscopic test and serves to detect the presence of as low a content as 5 per cent of carbon monoxide hemoglobin.

Alkali Test.—Mix two drops of the suspected blood on a porcelain plate with an equal volume of 25 per cent NaOH. A reddish color remains in the presence of carbon monoxide hemoglobin. Treat two drops of normal blood in the same way. A brownish color is obtained.

Quantitative Determination of Carbon Monoxide.[4]—Wrap a finger of the subject with a rubber band. Prick deeply with a lancet. Draw 0.1 c.c. of blood into a pipette. Discharge into a test tube containing 0.4 c.c. of dilute ammonia (1 c.c. of strong ammonia to one liter of water). Mix immediately but do not shake. Add 1.5 c.c. of a freshly prepared 1 per cent tannic acid solution and mix. Prepare at the same time a series of standards as follows. Saturate 5 c.c. of normal human or dog's blood with CO by bubbling through it illuminating gas until completely in foam. Lake 1 c.c. of this blood and also 1 c.c. of the normal untreated blood with dilute ammonia. Mix proportional parts of these laked bloods in a series of test tubes to represent 10, 20, 30, 40, 50, 60, and 100 per cent saturation. Add tannic acid to each. Keep unknowns and standards over night and compare.

[1] Sand (*Ugeskrift for Laeger*, 76, 1721, 1914; Abst. *J. A. M. A.*, Nov. 21, 1914) proposes a potassium iodide test for carbon monoxide hemoglobin in blood. He claims 0.125 per cent may be detected by his test.

[2] This transforms the oxyhemoglobin into methemoglobin.

[3] This is done to free the blood from carbon monoxide hemoglobin.

[4] Henderson and Haggard: *Jour. Am. Med. Assn.*, 79, 1143, 1922; See also Sayers and Yant: *Public Health Reports*, Reprint No. 790, Oct. 1922 and Sayers, Yant and Jones: *Public Health Reports*, 38, 2311, 1923; and especially Sayers and Yant: Bureau of Mines, *Technical Paper* 373, 1925. For gasometric determination of carbon monoxide in blood see pp. 457 and 468.

4. Neutral Methemoglobin.—Dilute a little defibrinated blood (1:10) and add a few drops of a freshly prepared 10 per cent solution of potassium ferricyanide. Shake this mixture and observe that the bright red color of the blood is displaced by a brownish red. Now dilute a little of this solution and examine it spectroscopically. Note the single, very dark absorption band lying to the left of D, and, if the dilution is sufficiently great, also observe the two rather faint bands lying between D and E in somewhat similar positions to those occupied by the absorption bands of oxyhemoglobin. Add a few drops of Stokes' reagent to the methemoglobin solution while it is in position before the spectroscope and note the immediate appearance of the oxyhemoglobin spectrum which is quickly followed by that of hemoglobin.

5. Alkaline Methemoglobin.—Render a neutral solution of methemoglobin, such as that used in the last experiment (4), slightly alkaline with a few drops of ammonia. The solution becomes redder in color, due to the formation of alkaline methemoglobin and shows a spectrum different from that of the neutral body. In this case we have a band on either side of D, the one nearer the red end of the spectrum being much the fainter. A third band, darker than either of those mentioned, lies between D and E somewhat nearer E.

6. Alkali Hematin.—Observe the spectrum of the alkali hematin prepared in Experiment 18 on page 352. Also make a spectroscopic examination of a freshly prepared alkali hematin.[1] The typical spectrum of alkali hematin shows a single absorption band lying across D and mainly toward the red end of the spectrum.

7. Reduced Alkali Hematin or Hemochromogen.—Dilute the alkali hematin solution used in the last experiment (6) to such an extent that it shows no absorption band. Now add a few drops of Stokes' reagent or ammonium sulphide and note that the greenish-brown color of the alkali hematin solution is displaced by a bright red color. This is due to the formation of hemochromogen or reduced alkali hematin. Examine this solution spectroscopically and observe the narrow, dark absorption band lying midway between D and E. If the dilution is not too great a faint band may be observed in the green extending across E and b.

8. Acid Hematin.—To some defibrinated blood add half its volume of glacial acetic acid and an equal volume of ether. Mix thoroughly. The acidified ethereal solution of hematin rises to the top and may be poured off and used for the spectroscopic examination. If desired it may be diluted with acidified ether in the ratio of one part of glacial acetic acid to two parts of ether. A distinct absorption band will be noted in the red between C and D and lying somewhat nearer C than the band in the methemoglobin spectrum. Between D and F may be seen a rather indistinct broad band. Dilute the solution until this band resolves itself into two bands. Of these the more prominent is a broad, dark absorption band lying in the green between b and F. The second, a narrow band of faint outline, lies in the light green to the red side of E. A fourth very faint band may be observed lying on the violet side of D.

9. Acid Hematoporphyrin.—To 5 c.c. of concentrated sulphuric acid in a test-tube add 2 drops of blood, mixing thoroughly by agitation after the addition of each drop. A wine-red solution is produced. Examine this solution spectroscopi-

[1] Alkali hematin may be prepared by mixing one volume of a concentrated potassium hydroxide or sodium hydroxide solution and two volumes of dilute (1 : 5) defibrinated blood. This mixture should be heated gradually almost to boiling, then cooled and shaken for a few moments in the air before examination.

cally. Acid hematoporphyrin gives a spectrum with an absorption band on either side of D, the one nearer the red end of the spectrum being the narrower.

10. **Alkaline Hematoporphyrin.**—Introduce the acid hematoporphyrin solution just examined into an excess of distilled water. Cool the solution and add potassium hydroxide slowly until the reaction is but slightly acid. A colored precipitate forms which includes the principal portion of the hematoporphyrin. The presence of sodium acetate facilitates the formation of this precipitate. Filter off the precipitate and dissolve it in a small amount of dilute potassium hydroxide. Alkaline hematoporphy in prepared in this way forms a bright red solution and possesses four absorption bands. The first is a very faint, narrow band in the red, midway between C and D; the second is a broader, darker band lying across D, principally to the violet side. The third absorption band lies principally between D and E, extending for a short distance across E to the violet side, and the fourth band is broad and dark and lies between b and F. The first band mentioned is the faintest of the four and is the first to disappear when the solution is diluted.

CHAPTER XIX

RESPIRATORY METABOLISM AND NEUTRALITY REGULATION

The two vital processes which take place in the lungs during respiration are the addition of oxygen to the blood, and the removal of carbon dioxide from the blood. The oxygen is carried to all the tissues of the body where it is utilized in the metabolic processes taking place within the cells. Among the end products of cell metabolism are certain acids, organic and inorganic, chief of which is carbonic acid. The other acids, like phosphoric, sulphuric and lactic, are for the most part neutralized as soon as they are formed and exist in the blood as salts. The base for this neutralization is supplied by sodium bicarbonate, the result being the formation of additional carbonic acid. Hence, it is evident that the latter is not only an important end product of tissue oxidation but also plays a significant role in neutrality regulation. The store of sodium bicarbonate in the blood (alkali reserve) must obviously never fall too low to meet the demands of acid production by the tissues. The capacity of the carbonic acid: sodium bicarbonate system to control the neutrality of the blood depends on the volatility of carbon dioxide and hence its elimination by the lungs. The non-volatile acids (chiefly in the form of their salts) such as phosphoric, lactic and in pathological conditions, acetoacetic and β-hydroxybutyric acids, are disposed of by the kidneys.

An understanding of the mechanism of neutrality regulation is impossible without a knowledge of how these gases, O_2 and CO_2, exist in and are transported by the blood. Hemoglobin, a so-called respiratory pigment, exists normally in the blood in two forms, *reduced hemoglobin*, and in loose combination with oxygen, as *oxyhemoglobin*. The latter is in equilibrium with oxygen in the physically dissolved state, and when this is disturbed, as by diffusion of O_2 outward, the concentration of oxyhemoglobin changes. When venous blood comes to the lungs, it is distributed in the alveoli, exposing a great surface of contact to the air. The higher O_2 tension in the latter causes more oxygen to be dissolved in the blood and combination with reduced hemoglobin rapidly ensues. When the aerated blood leaves the lungs almost all of the hemoglobin is in the form of oxyhemoglobin. The protein oxyhemoglobin ($HHbO_2$), however, behaves as a slightly stronger acid than

reduced hemoglobin (HHb), and hence accompanying its formation some CO_2 is set free from sodium bicarbonate. Thus oxygenation of hemoglobin facilitates the elimination of CO_2. In the tissues the reverse change takes place. The blood gives up oxygen to the tissues as it takes up the waste CO_2, part of which is neutralized by the base liberated from oxyhemoglobin as the latter is converted to the weaker acid, reduced hemoglobin. Since this neutralization causes no alteration in the hydrogen ion concentration of the blood, the more basic form of hemoglobin being balanced by the acid CO_2, it is termed the *isohydric* change. In this indirect manner hemoglobin plays a role in the transport of CO_2 in addition to its function as an O_2-carrier.

As has been indicated, the isohydric change accounts for only part of the CO_2-carrying power of the blood. When more CO_2 enters than can be neutralized in this manner, the blood becomes more acid, *i.e.* its hydrogen ion concentration increases. This disturbs the equilibria of all the buffer systems, shifting the ratios of salt to acid in the direction of more acid. Thus in the ratios

$$\frac{BHb}{HHb} \qquad \frac{BHbO_2}{HHbO_2} \qquad \frac{B_2HPO_4}{BH_2PO_4} \qquad \frac{BProtein}{HProtein}$$

the diminution in the salt concentrations (numerators) results in the liberation of base, with which CO_2 combines. Not only the inorganic buffers but also the proteins and organic acids enter into this phase of CO_2 transport. Hemoglobin, it will be seen, acts in a double capacity, namely isohydrically and as a buffer.

The buffers of the blood, being salts of weak acids, are able to take up limited amounts of acid with much less change in hydrogen ion concentration than would result from the same addition of acid to water, or sodium chloride solution, which have no buffer power. The buffer systems above referred to act therefore as reservoirs of alkali for the neutralization of the acid end-products of metabolism. It can be proven mathematically and demonstrated experimentally that the efficiency of a buffer system in resisting change in reaction is greatest when the ratio of salt to acid is equal to one. Fig. 127 represents a titration curve for a weak acid (carbonic), that is, a curve in which the fraction of total acid neutralized is plotted against the reaction at each step in the titration. The S-shaped curve is characteristic of buffer acids or alkalis, and demonstrates that in the middle portion of the curve, where half of the acid is neutralized (ratio of salt to acid $= 1$) the slope (rate of change of pH) is least.

The relation between the reaction (pH) and the ratio of buffer acid (HA) to buffer salt (BA) is derived as follows. The equation for the

electrolytic dissociation of a weak acid into hydrogen ion (H^+) and the anion (A^-) is $HA \leftrightarrow H^+ + A^-$. From the law of mass action we know that the velocity of this reversible reaction in either direction is proportional to the concentrations of the reacting constituents. That is,

$$\text{Velocity (left to right)} = k_1 HA$$

and

$$\text{Velocity (right to left)} = k_2(H^+ \times A^-),$$

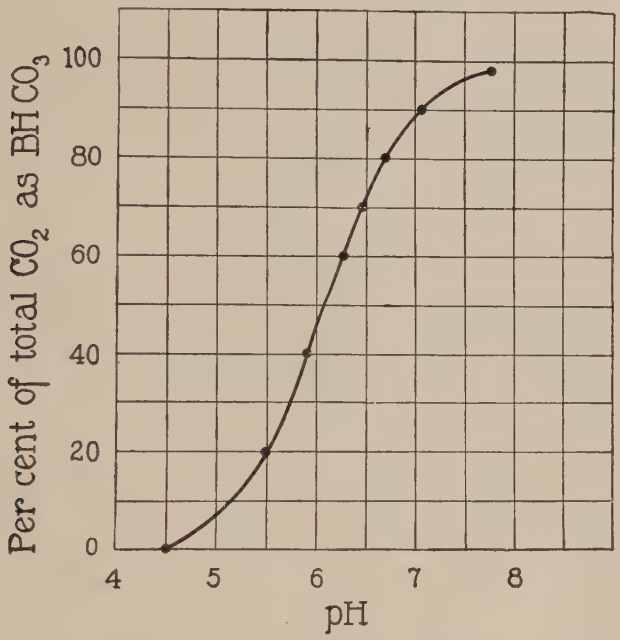


FIG. 127.—TITRATION CURVE FOR THE H_2CO_3:$BHCO_3$ SYSTEM.
(*From Van Slyke, in "Endocrinology and Metabolism," vol. 4, Appleton,* 1924.)

in which the symbols also represent concentrations. At a condition of equilibrium the rates of reaction in each direction are the same, the equilibrium being dynamic. We may therefore write

$$k_1 HA = k_2(H^+ \times A^-)$$

or transposing,

$$\frac{k_1}{k_2} = \frac{(H^+ \times A^-)}{HA} = K$$

since one constant divided by another gives a third constant. K is known as the dissociation constant of the acid. Hence

$$H^+ = K \times \frac{HA}{A^-}.$$

In the case of a weak acid and its salt, however, only a very small fraction of the anion, A^-, originates from the dissociation of the free acid,

practically all of it coming from the dissociation of the salt, BA, into B^+ and A^-. Most salts in the concentration found in body fluids are ionized to the extent of 60 to 90 per cent. "If the degree of dissociation be represented by λ, the concentration of anions, $A^- = \lambda BA$ and we may rewrite the last equation

$$H^+ = K \times \frac{HA}{\lambda BA}.$$

Since λ varies to a relatively slight extent over ranges of concentration within such limits as are found in blood constituents, one may state as a close approximation that

$$\frac{K}{\lambda} = K_1$$

and

$$H^+ = K_1 \frac{HA}{BA}.$$

By definition, $pH = -\log H^+$ (see p. 9). (For example, the hydrogen ion concentration $[H^+]$ at neutrality is 0.000.000.1 normal $= 10^{-7}$. Therefore the $pH = -\log 10^{-7} = -(-7) = 7$.) Hence, taking the logarithms of both sides of the equation,

$$\log H^+ = \log K_1 + \log \frac{HA}{BA}$$

and, changing signs, $pH = -\log K_1 - \log \frac{HA}{BA}$

or

$$pH = pK_1 + \log \frac{BA}{HA}.$$

The value of the constant pK_1 ($-\log K_1$) for various acids is numerically equal to the pH when the ratio of salt BA to acid HA is unity, since the $\log 1 = 0$. Under this condition, it will be recalled, the maximum efficiency of the buffer action obtains. The following table gives the pK_1 values for the most important buffers in blood.

Buffer system	pK_1
$BHCO_3 : H_2CO_3$	6.1
$B_2HPO_4 : BH_2PO_4$	6.8
$BHbO_2 : HHbO_2$	7.16
$BHb : HHb$	7.3

It is significant that the pH of maximum efficiency of these buffers, particularly the phosphate and bicarbonate systems, is below the normal pH for blood, since as the blood pH falls, change of reaction is opposed with an efficiency which increases as the danger point is approached.

Serum separated from the red corpuscles ("separated serum") has only a slight buffer effect and consequent CO_2-carrying power. But

while yet in contact with the cells ("true serum"), the serum approximates the whole blood in this respect. This is due to the capacity of the cells for transmitting their buffer properties to the serum through the passage of CO_2, O_2 and HCl across the cell membrane. The mechanism by which this takes place is illustrated by the following scheme.

Plasma	Red cell wall	Cell
$H_2CO_3 + Na\ Protein \leftrightarrow H\ Protein + NaHCO_3$ $H_2CO_3 + NaCl \leftrightarrow NaHCO_3 + HCl$ ←——→	$HCl \leftrightarrow$	$HCl + K_2HPO_4 \leftrightarrow KH_2PO_4 + KCl$ $2HCl + 2KHbO_2 \leftrightarrow 2KCl +$ { $HHbO_2$ / $HHb + O_2$ ←—
H_2CO_3 ←——————→	$H_2CO_3 \leftrightarrow$	$H_2CO_3 + K_2HPO_4 \leftrightarrow KHCO_3 + KH_2PO_4$ $2H_2CO_3 + 2KHbO_2 \leftrightarrow 2KHCO_3 +$ { $HHbO_2$ / HHb + O_2 ←—
O_2 ←——————	—O_2—	

It will be noted that no base (cation) permeates the cell membrane. The passage of chloride ion into the cells as the carbonic acid (CO_2 tension) of the plasma is increased, and the reverse when it is decreased, is known as the "chloride shift." By virtue of this exchange, the cells act as reservoirs of buffer alkali to maintain constancy of reaction in the plasma, although the buffers themselves (hemoglobin and phosphates) do not leave the cells.

The redistribution of Cl^-, HCO_3^- and H^+ (or OH^-) between plasma and cells which accompanies change in oxidation and reduction of hemoglobin involves an alteration in the osmotic relations within and without the cells, and a subsequent water shift. This relationship and the part played by the Donnan equilibrium (p. 4) have been reviewed by Van Slyke, Wu and McLean.[1]

The changes that normally take place in blood in the transition from the arterial to the venous state, are summarized in the following table, adapted from the data of Henderson, Bock, Field and Stoddard.[2]

[1] Van Slyke, Wu and McLean: *Jour. Biol. Chem.*, 56, 765, 1923. For a fuller treatment of the quantitative relations concerned in the transport of CO_2 by the blood, the reader is referred to the reviews by Wilson: *Physiol. Rev.*, 3, 295, 1923; Van Slyke: *Ibid.*, 1, 141, 1921; and Austin and Cullen: *Medicine*, 4, 275, 1925.

[2] *Jour. Biol. Chem.*, 59, 379, 1924.

	Arterial blood			Venous blood		
	Whole blood	Serum	Cells	Whole blood	Serum	Cells
pH	....	7.45	7.31	....	7.42	7.30
Free CO_2 vol. per cent of whole blood	2.4	1.6	0.8	2.7	1.8	0.9
Combined CO_2 vol. per cent of whole blood	45.8	34.0	11.8	50.4	36.8	13.6
NaCl, mg. per 100 c.c. whole blood	468	349	119	468	342	126
Cell volume, per cent	....	....	40.0	....	....	40.5
Combined O_2, vol. per cent of whole blood	19.3	.....	.....	13.0		
Saturation of Hb, per cent	96.0	.....	.....	65.0		

From this table it will be seen (1) that venous blood is slightly more acid than arterial, due to its relatively higher proportion of free CO_2 (H_2CO_3) to combined CO_2 ($BHCO_3$); (2) that chlorides shift from serum to cells as the CO_2 tension of the blood increases; (3) that in consequence of this shift of ions, the osmotic relationships are disturbed (Donnan equilibrium) and water diffuses into the cells, increasing their relative volume, (4) that arterial blood loses part of its oxygen in the tissues, in other words, becomes unsaturated.

An idea as to the distribution of the load of CO_2 among the various buffers and hemoglobin when arterial blood takes on carbon dioxide in the tissues and becomes venous, may be obtained from the following table, which represents a quantitative study of a case presented by Doisy, Briggs, Eaton and Chambers.[1]

CARBON DIOXIDE CARRIED BY BUFFER SYSTEMS STUDIED, WHEN ARTERIAL BLOOD (pH 7.31) CHANGES TO VENOUS BLOOD (pH 7.28).

	Vol. per cent	Per cent of total
Total CO_2 carried for R. Q. of 0.75	4.23	
$BHCO_3$ carried isohydrically $BHbO_2 \leftrightarrow HHb$	2.26	53.5
$BHCO_3$ carried by change of pH (Buffers):		
$BHbO_2 : HHbO_2$ / $BHb : HHb$	1.07	25.3
$B_2HPO_4 : BH_2PO_4$ in cells	0.01	0.3
Separated serum	0.20	4.7
CO_2 physically dissolved	0.51	12.1
Sum, per cent of total	4.05	96.0
Per cent of total CO_2 carried by hemoglobin	78.8	

[1] *Jour. Biol. Chem.*, 54, 305, 1922.

Due to the ease of elimination of CO_2 by respiration, the regulation of the neutrality of the blood is largely controlled by the $H_2CO_3 : BHCO_3$ system. By means of variation in the rate and depth of respiration and the rate of blood flow through the lungs, opportunity is afforded for very delicate adjustment of the hydrogen ion concentration. The nervous control of the respiratory mechanism resides in the respiratory center in the medulla. Whether the stimulus to the center is a specific property of the blood, and whether this property is the CO_2 tension or the hydrogen ion concentration, are still unsettled questions. Increases in either factor call forth corresponding responses in the ventilation of the lungs, and since changes in CO_2 and hydrogen ion concentration take place concurrently, it is difficult to consider them separately. It is probable that increases in hydrogen ions in the respiratory center itself, secondary to those of the blood, may be the activating factor. Such increases may come about through diffusion of CO_2 or H^+ from the blood or by acid production within the center. The latter might explain the greater respiratory response which results from an insufficient oxygen supply to the center.

The rate of elimination of waste products through the lungs and the kidneys is partly controlled by the rate of blood flow through those organs. The diminished blood pressure following excessive pulmonary ventilation has been definitely shown to be due to the loss of CO_2.[1] The accompanying rise in pH is not of itself the factor which produces the heightened blood pressure. There is, however, no constant relation between blood pressure and CO_2 tensions among different individuals.

The role of the kidneys in neutrality regulation is concerned chiefly with the elimination of non-volatile acids, and to a minor degree, of bases. In the body the stronger acids react with buffers, chiefly the phosphates, according to the reaction:

$$B_2HPO_4 + HCl = BH_2PO_4 + BCl.$$

The major portion of acid phosphate which is excreted represents an equivalent amount of neutralized acid. β-Hydroxybutyric acid, an abnormal acid product of fat metabolism associated with diabetes, is excreted mainly in the form of the salt, most of the base for which comes from, and hence depletes, the alkali reserve in the blood. An excess of base is eliminated through the kidneys as disodium phosphate and sodium bicarbonate.

An important mechanism in the elimination of acids is the ammonia forming function of the kidneys[2] by virtue of which base is spared to

[1] Dale and Evans: *Jour. Physiol.*, 56, 125, 1922.

[2] Nash and Benedict: *Jour. Biol. Chem.*, 48, 463, 1921 and 51, 183, 1922. Benedict and Nash: *Jour. Biol. Chem.*, 69, 381, 1926. Rabinowitch, *Jour. Biol. Chem.*, 69, 283, 1926.

the blood. It is claimed[1] however that the kidney is not the sole seat of ammonia production, but that the latter is a generalized tissue phenomenon.

The normal reaction of the urine ranges from pH 4.8 to 7.4. While the pH of the urine serves as an index of the acid output, a measure of the quantity excreted can be gained only by titration, and preferably to the pH of the blood. By this method the "physiological elimination of acids," *i.e.*, the amount of acid excreted in excess of the normal blood reaction, may be estimated.

Inasmuch as the chief factor in the regulation of blood reaction is the H_2CO_3:$BHCO_3$ system, the relationship of variations in the latter pH should yield valuable information concerning changes in the acid-base balance of the body. Applying the equation for the dissociation of a weak acid (see page 421) to this case, the so-called Hasselbalch equation is obtained.

$$pH = pK_1 + \log \frac{BHCO_3}{H_2CO_3}$$

The value of the constant pK_1 for blood plasma has been ascertained as 6.10. By measuring any two of the remaining variables, the third is of course determined. If we plot changes in bicarbonate concentration [$BHCO_3$] as ordinates, against carbonic acid [H_2CO_3] as abscissae, a straight line will result for any given ratio of the two, and hence for any given pH. The slope of this line will increase or decrease with corresponding changes in the [$BHCO_3$]:[H_2CO_3] ratio. The maximal as well as the normal ranges of these factors are depicted in Fig. 128, constructed by Van Slyke.[2] "Since it was desired to use the customary form of CO_2 absorption curves, with total CO_2 values, [$BHCO_3$ + H_2CO_3], as ordinates, and CO_2 tensions as abscissae," the coordinates are expressed in terms of these factors, but the same relation between them still obtains. It will be noted that nine areas are set off as a result of three possible variations (high, normal or low) in each of two variables. This graph has been modified to express changes in [$BHCO_3$] with respect to pH, Fig. 129, since these are the factors usually measured. The nine areas represent conditions of acid of alkali excess or deficit, which are either *compensated* (pH normal) or *uncompensated* (pH above or below normal). That is, a condition of alkali deficit (low alkali reserve) may be either compensated by a corresponding diminution in carbonic acid so that the [$BHCO_3$]:[H_2CO_3] ratio remains normal (about 20:1), or it may be uncompensated by the failure to remove sufficient CO_2 in which case the pH falls and the condition of uncompen-

[1] Bliss: *Jour. Biol. Chem.*, 67, 109, 1926.
[2] Van Slyke: *Jour. Biol. Chem.*, 48, 153, 1921.

sated alkali deficit results. It is therefore obvious that "acidosis" can not be regarded as merely a lowering of the alkali reserve, for such a

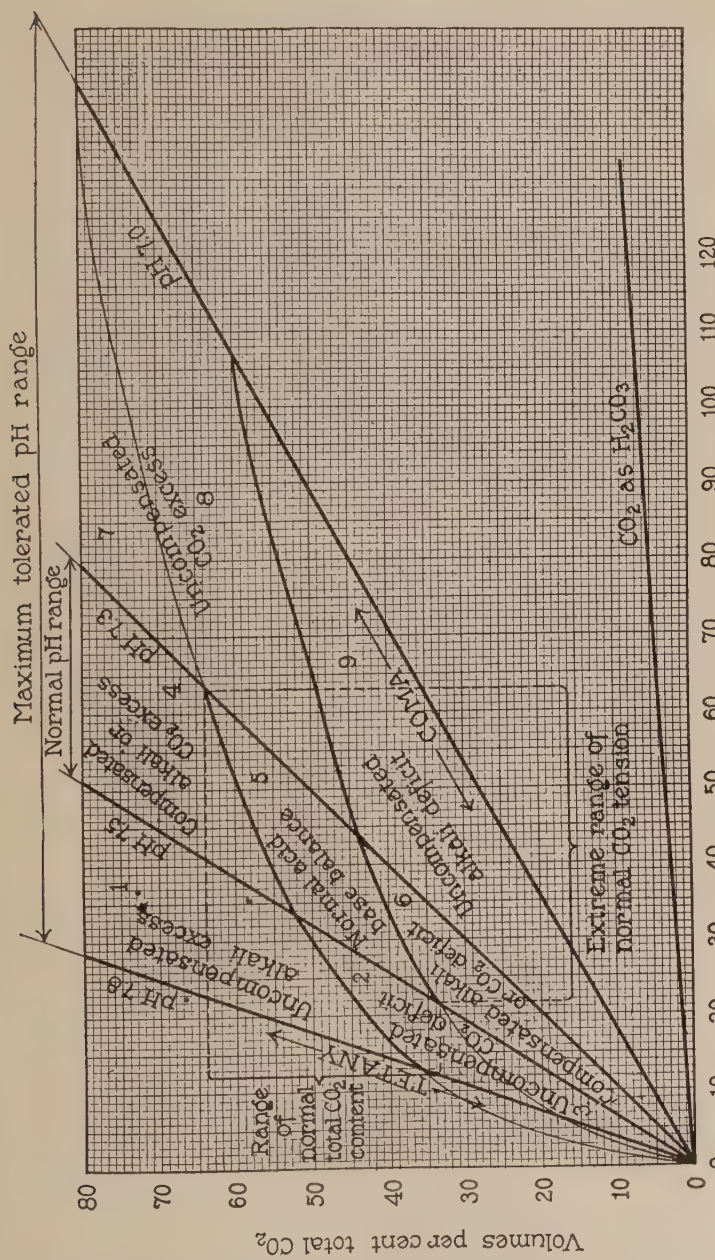


FIG. 128.—NORMAL AND ABNORMAL VARIATIONS OF THE $BHCO_3$, H_2CO_3, CO_2 TENSION, AND pH IN OXYGENATED HUMAN WHOLE BLOOD DRAWN FROM RESTING SUBJECTS AT SEA LEVEL. (*Van Slyke.*)
The bicarbonate CO_2 at any point is obtained by subtracting from the total CO_2 the relatively small amount present as H_2CO_3 indicated by the slanting line near the bottom of the figure.

condition may accompany a deficit of CO_2 so marked as to result in a more *alkaline* reaction of the blood. Moreover an abnormally *acid reaction* may occur concomitant with an *increase of alkali reserve*

provided CO_2 is present to excess. In order therefore to combine the effects of both alkali reserve and reaction, Van Slyke has broadly defined acidosis as a condition caused "by the formation or absorption of acids at a rate exceeding that of their elimination . . . (which) . . . may be considered to have caused an abnormal state when it has

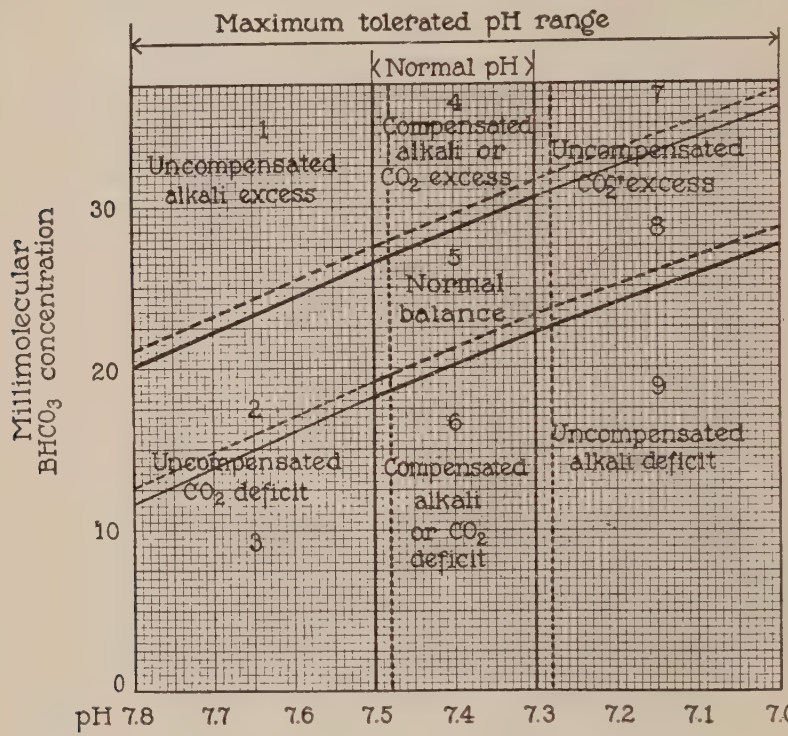


FIG. 129.—NORMAL AND ABNORMAL VARIATIONS OF THE $BHCO_3$ AND pH IN SERUM OR OXALATED PLASMA. (*Van Slyke.*)

The arterial conditions are indicated by the solid curves, venous by the broken curves. $BHCO_3$ and pH values are represented with rectangular coordinates, and the diagram as compared with Fig. 128, is simplified by omitting H_2CO_3 and CO_2 tension values. $BHCO_3$ values are expressed in terms of millimolecular concentration (1 millimol $BHCO_3$ = 2.24 volumes per cent of bicarbonate CO_2). The curves are 4 millimols higher than for whole blood, since $BHCO_3$ concentration in the plasma at any given pH is higher than that of the whole blood by approximately 10 vol. per cent of bicarbonate CO_2 or 4 millimols per liter.

either increased the hydrion concentration of the blood or lowered its alkali reserve below the extreme normal limit."

The conditions which fall into the different areas have been observed both clinically and experimentally, and have been reviewed by Van Slyke,[1] Myers and Booher[2] and Austin and Cullen.[3]

In the following table the more important conditions associated with disturbance of acid-base balance are given, together with their causes

[1] Van Slyke: *Jour. Biol. Chem.*, 48, 153, 1921.
[2] Myers and Booher: *Jour. Biol. Chem.*, 59, 699, 1924.
[3] Austin and Cullen: *Medicine*, 4, 275, 1925.

and the physiological mechanisms brought into play for their compensation.

DISTURBANCES OF ACID-BASE EQUILIBRIUM OF BLOOD

Area	Acid-base balance	Conditions	Associated symptoms	Compensatory mechanisms
1 Uncompensated Alkali Excess	($BHCO_3$) increased without proportionate rise in (H_2CO_3), therefore pH increased.	Overdosage of $NaHCO_3$. Excessive vomiting (pyloric obstruction) or gastric lavage (loss of HCl). X-ray treatment.	If marked, tetany.	Diminished respiration (rise in alveolar CO_2) to hold back CO_2. Diuresis and increased $NaHCO_3$ excretion.
2–3 Uncompensated CO_2 Deficit	(H_2CO_3) decreased without proportionate fall in ($BHCO_3$), therefore pH increased.	Hyperpnea, voluntary or induced (oxygen want, *e.g.* at high altitudes). Fever. Hot baths.	If marked, tetany.	Retention of acid metabolites (low NH_3 and titratable acidity of urine). Excretion of $NaHCO_3$.
4 Compensated Alkali or CO_2 Excess	Either ($BHCO_3$) or (H_2CO_3) increased but balanced by proportionate rise in other constituent, therefore pH normal.	*Alkali excess.* $NaHCO_3$ therapy, with slow absorption. *CO_2 excess.* Retarded gas exchange (*e.g.* emphysema) with CO_2 tension chronically increased.	Cyanosis due to deficient oxygen exchange.	CO_2 retention. $BHCO_3$ retention.
5 Normal	($BHCO_3$) and (H_2CO_3) normal at ordinary altitudes.			
6 Compensated Alkali or CO_2 Deficit	Either ($BHCO_3$) or (H_2CO_3) decreased but balanced by proportionate fall in other constituent, therefore pH normal.	*Alkali deficit.* Accelerated production (*e.g.*, diabetes) or retarded elimination (*e.g.* nephritis) of nonvolatile acids. Experimental acid intoxication. Diarrheal acidosis of infancy (marasmus). *CO_2 deficit.* Overventilation at high altitudes (oxygen want).	Hyperpnea.	Increased respiration ("blowing off CO_2"). Accelerated NH_3 formation and acid excretion. Same as in Areas 2 and 3.
7–8 Uncompensated CO_2 Excess	(H_2CO_3) increased without proportionate rise in ($BHCO_3$), therefore pH decreased.	Retarded respiration as in pneumonia (physical obstruction) or morphine narcosis (deadening of respiratory center). Experimental re-breathing. Cardiac decompensation.	Dyspnea.	Increased respiration. Accelerated NH_3 formation and acid excretion. Probable shift of acid from blood to tissue.
9 Uncompensated Alkali Deficit	($BHCO_3$) decreased without proportional fall in (H_2CO_3), therefore pH decreased.	Terminal stages of nephritic acidosis, and diabetic acidosis (compensated by insulin therapy). Deep ether anesthesia. Certain cardiac cases.	Dyspnea.	Increased respiration. Increased acid excretion and NH_3 formation (except probably in nephritis).

Of the acetone bodies, the acetoacetic acid is considered to be the most important. This acid has its origin principally in fats, and to a minor degree in certain amino acids resulting from protein cleavage.

It has been demonstrated that acetoacetic acid may be formed in the body through the oxidation of butyric acid, and that the administration of fats containing butyrin to diabetics causes an increased production of acetoacetic acid. Furthermore, it is believed that fatty acids higher than butyric acid in the series also yield acetoacetic acid by oxidation. In this change the oxidation occurs at the β-carbon, two carbon atoms at a time being involved. As soon as the oxidation proceeds to the butyric acid stage this acid is transformed into acetoacetic acid. In diabetes the body either does not possess the normal power of oxidizing acetoacetic acid or else this acid is produced in excessive amount. At any rate, we find it in blood and urine in abnormal quantity. The relationship of acetoacetic acid to fatty acids may be expressed as follows:

$$\underset{\textit{Caproic acid}}{CH_3\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH_2\cdot COOH}$$
$$\downarrow O$$
$$CH_3\cdot CH_2\cdot CH_2\cdot CO\ CH_2\cdot COOH$$
$$\downarrow O$$
$$\underset{\textit{Butyric acid}}{CH_3\cdot CH_2\cdot CH_2\cdot COOH}$$
$$\downarrow O$$
$$\underset{\textit{Acetoacetic acid}}{CH_3\cdot CO\cdot CH_2\cdot COOH}$$

The β-hydroxybutyric acid is formed from acetoacetic acid by *reduction*. It was originally believed that this procedure was reversed and that the acetoacetic acid was formed from the β-hydroxybutyric acid by *oxidation*. However, it has been shown that the introduction of β-hydroxybutyric acid into the body is not followed by an increased acetoacetic acid formation, whereas, β-hydroxybutyric acid is formed when acetoacetic acid is introduced. Therefore, it seems clear that the acetoacetic acid is the original substance from which the β-hydroxybutyric acid is formed by a process of reduction. The relationship between the acetone bodies may be indicated in this way:

$$\underset{\textit{Acetoacetic Acid}}{CH_3\cdot CO\cdot CH_2\cdot COOH} \rightarrow \underset{\textit{Acetone}}{CH_3\cdot CO\cdot CH_3} + CO_2$$
$$\downarrow H \text{ (reduction)}$$
$$\underset{\textit{β-hydroxybutyric acid}}{CH_3\cdot CHOH\cdot CH_2\cdot COOH}$$

In the normal body it is probable that the bulk of the acetoacetic acid is oxidized to acetic acid and carbonic acid in turn and thence to carbon dioxide and water, whereas, in the diabetic organism this does not occur, at least not to any great extent. Likewise in the absence of carbohydrate in the diet, the oxidation is not complete and acetone bodies are increased in amount in both blood and urine. Thus "fat burns in the flame of carbohydrate, but without it, it smokes." The phenomenon of excessive acetone body production (ketosis) as it appears

in the human organism is not entirely the same as in other species, such as the rat, dog, rabbit, goat, pig and cow.

The effects of carbohydrate, protein and fat in stimulating the formation of acetone bodies (*ketogenesis*) or inhibiting it (*antiketogenesis*) are discussed in Chapter XXXII under Experiments on Acid-Base Metabolism.

The appearance of the "acetone bodies," *i.e.*, acetone, acetoacetic acid and β-hydroxybutyric acid *in the urine* in appreciable quantity was originally taken as the index of an "acidosis" and the extent of the acidosis was judged by the estimation of the amount of these bodies present in the urine. That this is not a reliable index is shown by the occasional observation of a pronounced "acidosis" with no appreciable increase in urinary acetone bodies. A high urinary ammonia coefficient (ammonia N:total N) was also early looked upon as an indication of "acidosis." However, this factor is not very useful in diagnosis in spite of the fact that the majority of such cases show a high urinary ammonia value. Certain dietetic changes may produce high urinary ammonia, therefore, it is not necessarily indicative of "acidosis." It is also true that fatal "acidosis" has been observed in uremia, and in nutritional disorders of infants, with no pronounced increase in the ammonia coefficient. In this connection, it is possible that in renal disability, the ammonia-forming function of the kidneys[1] is diminished.

With the development of blood analysis the content of these acetone bodies in blood plasma was looked to as an aid in the determination of the extent of "acidosis." But here again the hope of the clinician failed to materialize. It is the consensus of the best opinion at the present time that cases of disturbed acid-base balance can be best diagnosed and their course followed not by the determination of acetone bodies in either urine or blood but by the determination of certain other factors which are more or less typical of "acidosis." These include the following:

1. The determination of the "alkali reserve" of the blood.
2. The determination of the alkali tolerance of the patient.
3. The determination of the carbon dioxide tension of the alveolar air.
4. The determination of the hydrogen ion concentration of the blood.

Methods

1. Simple Demonstration of the Presence of Carbon Dioxide in Expired Air.—Into each of two small flasks or large test tubes introduce 25 c.c. of a clear saturated solution of barium hydroxide. After an ordinary inspiration, expire through a bent glass tube or pipette dipped beneath the surface of the solution in the first flask. Repeat the experiment with flask number two but hold the breath as long as possible after the inspiration before breathing out through the tube. Note the relative amounts of precipitate of barium carbonate

[1] Nash and Benedict: *Jour. Biol. Chem.*, 48, 463, 1921.
Benedict and Nash: *Jour. Biol. Chem.*, 69, 381, 1926. Rabinowitch, *Jour. Biol. Chem.*, 69, 283, 1926.

formed. To a third tube add 30 c.c. of water, one to two drops of barium hydroxide solution and a few drops of 0.04 per cent phenol red. Expire through the tube until a change takes place. What does this change indicate?

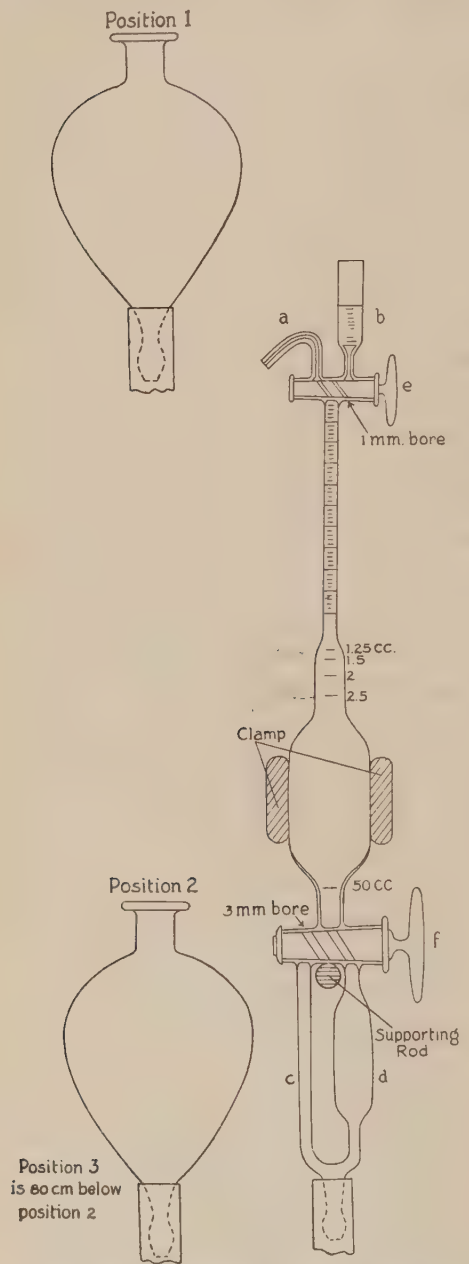


FIG. 130.—VAN SLYKE CARBON DIOXIDE APPARATUS. (*Journal Biological Chemistry*, 30, 289, 1917; 30, 347, 1917.)

2. Alkali Reserve.—Direct Method. (a) Carbon dioxide capacity of the plasma. (Van Slyke and Cullen.[1])

Principle.—The plasma from oxalated blood is shaken in a separatory funnel filled with an air mixture whose carbon dioxide tension approximates that of normal arterial blood, by which treatment it combines with as much carbon dioxide as it is able to hold under normal tension. A known quantity of the saturated plasma is then acidified within a suitable pipette, and its carbon dioxide is liberated by the production of a partial vacuum. The liber-

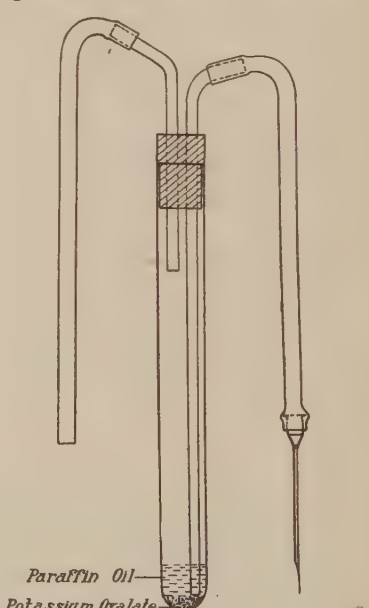


FIG. 131.—TUBE USED IN COLLECTING BLOOD. (*Journal Biological Chemistry*, 30, 289, 1917.)

[1] Van Slyke & Cullen: *Jour. Biol. Chem.*, 30, 289, 1917; Van Slyke: *Jour. Biol. Chem.*, 30, 347, 1917; Van Slyke and Stadie: *Jour. Biol. Chem.*, 49, 1, 1921.

ated carbon dioxide is then placed under atmospheric pressure, its volume carefully measured, and the volume corresponding to 100 c.c. of plasma calculated.

Apparatus.—The apparatus used in the estimation of the carbon dioxide content of the plasma is illustrated in Figs. 130 and 132. It is made of strong glass in order to stand the weight of mercury without danger of breaking, and is held in a strong screw clamp the

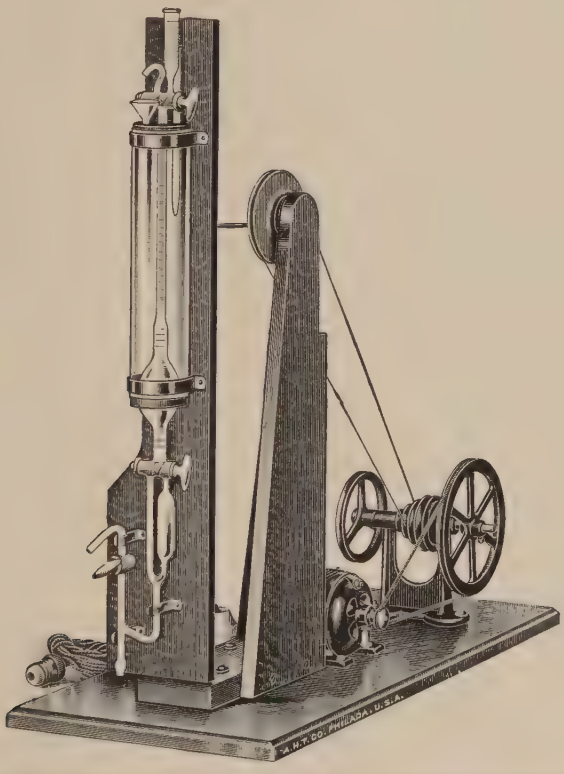


FIG. 132.—PRECISION MODEL OF VAN SLYKE BLOOD GAS ANALYSIS APPARATUS SHOWING WATER JACKET, SHOHL TRAP AND STADIE SHAKER.

jaws of which are lined with thick pads of rubber. In order to prevent accidental slipping of the apparatus from the clamp, an iron rod of 6 or 8 mm. diameter should be so arranged as to project under cock *f* between *c* and *d*.

Three hooks or rings at the levels 1, 2, and 3 serve to hold the levelling bulb at different stages of the analysis. The bulb is connected with the bottom of the apparatus by a heavy walled rubber tube.

It is necessary, of course, that both stop cocks should be properly greased and air tight, and it is also essential that they (especially *f*) shall be held in place so that they cannot be forced out by pressure of the mercury. Rubber bands may be used for this purpose but it is suggested that elastic cords of fine wire spirals, applied in the same manner as rubber bands, are stronger and more durable. Later models of this apparatus are equipped to overcome this difficulty.

After a determination has been finished, the levelling bulb is lowered without opening the upper cock, and most of the mercury is withdrawn from the pipette through *c*. The water solution from *d* is readmitted and the levelling bulb being raised to position 1, the water solution, together with a little mercury, is forced out of the apparatus through *a*.[1]

Procedure.—Drawing the blood.[2] **About six or seven c.c. of venous blood are aspirated into a centrifuge tube (see Fig. 131) which contains a little powdered potassium oxalate and some paraffin oil. The tube is subjected to a minimum of agitation after the blood is in it. The slight amount of agitation necessary to assure mixture with the oxalate is accomplished by stirring with the inlet tube, rather than by inverting or shaking. The tube and contents are then centrifuged.**

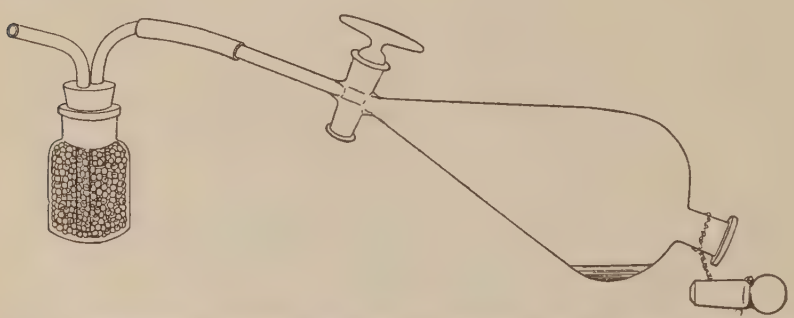


FIG. 133.—SEPARATORY FUNNEL USED IN SATURATING BLOOD PLASMA WITH CARBON DIOXIDE. (*Journal Biological Chemistry*, 30, 289, 1917.)

Saturation of Plasma with Carbon Dioxide.—After centrifugation about 3 c.c. of the plasma[3] **are transferred to a 300 c.c. separatory funnel, arranged as in Fig. 133, and the air within the funnel is displaced by either alveolar air**

[1] It is well to have a funnel draining into a special vessel to catch the water residues and mercury overflow from *a*. A considerable amount of mercury is thus regained if many analyses are run. It requires only straining through cloth or chamois skin to prepare it for use again.

[2] For at least an hour before the blood is drawn the subject should avoid vigorous muscular exertion as this, presumably because of the lactic acid formed lowers the bicarbonate of the blood. (Christiansen, Douglas & Haldane: *J. Physiol.*, 48, 246, 1914; Morawitz & Walker: *Biochem. Zeit.*, 60, 395, 1914.)

[3] If it is desired to keep the plasma for the estimation of carbon dioxide at a later time it should be transferred to a paraffined tube, covered with a layer of paraffin oil, stoppered and kept cold; under which conditions it is claimed that, if sterile, it may be kept for over a week without alteration of its carbon dioxide capacity.

from the lungs of the operator or a 5.5 per cent carbon dioxide-air mixture from a tank. In either case the gas mixture must be passed over glass beads before it enters the funnel.

When alveolar air is used the operator, without inspiring more deeply than normal, expires as quickly and as completely as possible through the glass beads and separatory funnel. The stopper of the funnel should be inserted just before the expiration is finished, so that there is no opportunity for air to be drawn back into the funnel. In order to saturate the plasma the separatory funnel is turned end over end for 2 minutes, the plasma being distributed in a thin layer as completely over the surface of the funnel's interior as is possible. After saturation is completed the funnel is placed upright and allowed to stand for a few minutes until the fluid has drained from the walls and gathered in the contracted space at the bottom of the funnel.

Determination of Carbon Dioxide.—The cup should be washed out with water and 1 c.c. of distilled water run in, and together with the entire apparatus should be filled with mercury to the top of the capillary tube by placing the levelling bulb of mercury in position 1. A sample of 1 c.c. (or 0.5 c.c. in case the amount of plasma available is very small) accurately pipetted, is allowed to run into the cup *b* in the apparatus represented in Fig. 130, the tip of the pipette remaining below the surface of the water as it is added. Add 1 drop of caprylic alcohol.[1]

With the mercury bulb at position 2 and the cock *f* in the position shown in the figure the plasma, water and alcohol are admitted from the cup into the 50 c.c. chamber, leaving just enough above the cock *e* to fill the capillary so that no air is introduced when the next solution is added. In the case of whole blood some corpuscles settle to the bottom of the cup. These are suspended in the water by stirring them up with the last 0.5 c.c. of water after the first 0.5 c.c. has passed into the chamber. Finally 0.5 c.c. of 5 per cent sulphuric acid is run in.[2]

It is not necessary that exactly 1 c.c. of wash water and 0.5 c.c. of acid shall be taken, but the total volume of the water solution introduced must extend exactly to the 2.5 c.c. mark on the apparatus, if the table on page 437 is to be used.

If the amount of plasma available is small a little more than 0.5 c.c. are saturated in a 50 c.c. funnel, and exactly 0.5 c.c. used for the estimation of carbon dioxide. In this case the volume of distilled water and acid used to wash the plasma into the apparatus is halved, so that the total volume of water solution introduced is only 1.25 c.c., and in the calculation the observed volume of gas is multiplied by 2.

After the acid has been added a drop of mercury is placed in *b* and allowed to run down the capillary as far as the cock in order to seal the latter. A copper wire may be used to expel a bubble of air which may be trapped in the capillary. Whatever excess of sulphuric acid remains in the cup is washed out with a little water, using a medicine dropper.

The mercury bulb is now lowered and hung at position 3 and the mercury in the pipette is allowed to run down to the 50 c.c. mark, producing a Torricellian

[1] It is desirable to keep the amount of caprylic alcohol small, as larger amounts may appreciably increase results. With plasma 0.02 c.c. is sufficient to prevent foaming and is measured most conveniently from a burette made by fusing a capillary stopcock to a pipette graduated into 0.01 c.c. divisions.

[2] With whole blood in place of sulphuric acid use lactic acid made by diluting 1 volume of concentrated acid (specific gravity 1.20) to 10 volumes with water.

vacuum in the apparatus. When the mercury (not the water) meniscus has fallen to the 50 c.c. mark the lower cock is closed and the pipette is removed from the clamp. Equilibrium of the carbon dioxide between the 2.5 c.c. of water solution and the 47.5 c.c. of free space in the apparatus is obtained by turning the pipette upside down fifteen or more times, thus thoroughly agitating the contents. The pipette is then replaced in the clamp.

After extraction of the gas the lower cock is opened admitting the mercury into the extraction chamber rapidly until the meniscus of the water solution reaches the contracted upper portion of the chamber. At this moment the lower cock is partially closed and the remainder of the mercury is admitted at a rate sufficiently retarded to prevent oscillation of the water column in the calibrated portion of the apparatus when pressure equilibrium is reached. The pressure is then adjusted by placing the mercury surface in the levelling bulb above the mercury meniscus in the chamber by a height equal to one-thirteenth that of the water column, in order to balance the latter. After some practice controlled by a centimeter rule one can estimate this level with the eye to within 2 mm. of mercury, which is sufficiently accurate for many purposes.[1] When the pressure has been adjusted the lower cock of the apparatus is closed. The gas volume may then be read at leisure.

When, as in most plasma analyses, the CO_2 is not reabsorbed no washing of the apparatus is necessary before using it for another determination, since the acid solution which wets the walls of the chamber contains a negligible amount of CO_2.

Calculation.—By means of the table on page 322 the readings on the apparatus can be directly transposed into c.c. of carbon dioxide chemically bound by 100 c.c. of plasma. The value obtained from this table must be multiplied by 1.017 to correct for CO_2 reabsorbed by the solution after release of pressure. The barometer reading and room temperature are taken at the time of the determination. For convenience in the calculation values are given below for the ratio $\frac{B}{760}$ over the range usually encountered.

Barometer	$\frac{B}{760}$	Barometer	$\frac{B}{760}$
732	0.963	756	0.995
734	0.966	758	0.997
736	0.968	760	1.000
738	0.971	762	1.003
740	0.974	764	1.006
742	0.976	766	1.008
744	0.979	768	1.011
746	0.981	770	1.013
748	0.984	772	1.016
750	0.987	774	1.018
752	0.989	776	1.021
754	0.992	778	1.024

[1] Stadie (*Jour. Biol. Chem.*, 49, 43, 1921) has suggested a leveling scale for more accurate reading as well as a convenient mechanical shaker for use in this method. (See Fig. 132.)

TABLE FOR CALCULATION OF CARBON DIOXIDE COMBINING POWER OF PLASMA[1]

Observed vol. gas $\times \frac{B}{760}$	C.c. of CO_2 reduced to 0° 760 mm. bound as bicarbonate by 100 c.c. of plasma				Observed vol. gas $\times \frac{B}{760}$	C.c. of CO_2 reduced to 0° 760 mm. bound as bicarbonate by 100 c.c. of plasma			
	15°	20°	25°	30°		15°	20°	25°	30°
0.20	9.1	9.9	10.7	11.8	0.60	47.7	48.1	48.5	48.6
1	10.1	10.9	11.7	12.6	1	48.7	49.0	49.4	49.5
2	11.0	11.8	12.6	13.5	2	49.7	50.0	50.4	50.4
3	12.0	12.8	13.6	14.3	3	50.7	51.0	51.3	51.4
4	13.0	13.7	14.5	15.2	4	51.6	51.9	52.2	52.3
5	13.9	14.7	15.5	16.1	5	52.6	52.8	53.2	53.2
6	14.9	15.7	16.4	17.0	6	53.6	53.8	54.1	54.1
7	15.9	16.6	17.4	18.0	7	54.5	54.8	55.1	55.1
8	16.8	17.6	18.3	18.9	8	55.5	55.7	56.0	56.0
9	17.8	18.5	19.2	19.8	9	56.5	56.7	57.0	56.9
0.30	18.8	19.5	20.2	20.8	0.70	57.4	57.6	57.9	57.9
1	19.7	20.4	21.1	21.7	1	58.4	58.6	58.9	58.8
2	20.7	21.4	22.1	22.6	2	59.4	59.5	59.8	59.7
3	21.7	22.3	23.0	23.5	3	60.3	60.5	60.7	60.6
4	22.6	23.3	24.0	24.5	4	61.3	61.4	61.7	61.6
5	23.6	24.2	24.9	25.4	5	62.3	62.4	62.6	62.5
6	24.6	25.2	25.8	26.3	6	63.2	63.3	63.6	63.4
7	25.5	26.2	26.8	27.3	7	64.2	64.3	64.5	64.3
8	26.5	27.1	27.7	28.2	8	65.2	65.3	65.5	65.3
9	27.5	28.1	28.7	29.1	9	66.1	66.2	66.4	66.2
0.40	28.4	29.0	29.6	30.0	0.80	67.1	67.2	67.3	67.1
1	29.4	30.0	30.5	31.0	1	68.1	68.1	68.3	68.0
2	30.3	30.9	31.5	31.9	2	69.0	69.1	69.2	69.0
3	31.3	31.9	32.4	32.8	3	70.0	70.0	70.2	69.9
4	32.3	32.8	33.4	33.8	4	71.0	71.0	71.1	70.8
5	33.2	33.8	34.3	34.7	5	71.9	72.0	72.1	71.8
6	34.2	34.7	35.3	35.6	6	72.9	72.9	73.0	72.7
7	35.2	35.7	36.2	36.5	7	73.9	73.9	74.0	73.6
8	36.1	36.6	37.2	37.4	8	74.8	74.8	74.9	74.5
9	37.1	37.6	38.1	38.4	9	75.8	75.8	75.8	75.4
0.50	38.1	38.5	39.0	39.3	0.90	76.8	76.7	76.8	76.4
1	39.1	39.5	40.0	40.3	1	77.8	77.7	77.7	77.3
2	40.0	40.4	40.9	41.2	2	78.7	78.8	78.7	78.2
3	41.0	41.4	41.9	42.1	3	79.7	79.6	79.6	79.2
4	42.0	42.4	42.8	43.0	4	80.7	80.5	80.6	80.1
5	42.9	43.3	43.8	43.9	5	81.6	81.5	81.5	81.0
6	43.9	44.3	44.7	44.9	6	82.6	82.5	82.4	82.0
7	44.9	45.3	45.7	45.8	7	83.6	83.4	83.4	82.9
8	45.8	46.2	46.6	46.7	8	84.5	84.4	84.3	83.8
9	46.8	47.1	47.5	47.6	9	85.5	85.3	85.2	84.8
0.60	47.7	48.1	48.5	48.6	1.00	86.5	86.2	86.2	85.7

[1] The temperature figures at the heads of columns represent the room temperature at which the samples of plasma are saturated with alveolar carbon dioxide and analyzed. It is assumed that both operations are performed at the same temperature. The figures have been so calculated that, regardless of the room temperature at which saturation and analysis are performed, the table gives the volume (reduced to 0°, 760 mm.) of carbon dioxide that 100 c.c. of plasma are capable of binding when saturated at 20° with carbon dioxide at approximately 41 mm. tension. If the figures in the table are multiplied by 0.04 they give, within 1 or 2 per cent of the carbon dioxide bound at 37°.

In case the volume of plasma taken for estimation of carbon dioxide content was 0.5 c.c. the observed volume of gas is multiplied by 2 before it is used to calculate the volume per cent of carbon dioxide bound.

Interpretation.—The carbon dioxide capacity of the plasma as determined by this method appears to indicate not only the alkaline reserve of the blood but also that of the entire body. The average normal value for man is 65 volume per cent of carbon dioxide. The table, page 439, shows the range of results obtained with normal and pathological plasma, as well as the relationship of the plasma bicarbonate to acid excretion, alkali tolerance, and alveolar carbon dioxide tension.

(b) **Plasma Bicarbonate (Titration Method) Van Slyke.**[1] *Principle.* Plasma is treated with an excess of standard acid which is titrated back with standard alkali under carefully standardized conditions.

Procedure.—In drawing and centrifugating the blood the precautions outlined by Cullen in his method for determining hydrogen ion concentration (for preventing loss or accumulation of CO_2 and consequent change in the distribution of bicarbonate between corpuscles and plasma) are to be observed. Oxalate plasma is used. Up to the beginning of the analysis, the blood and plasma are handled exactly as described for Cullen's method which may conveniently be combined with this determination. One c.c. of the plasma, to serve as a standard for the end-point, is treated exactly as described by Cullen to prepare it for colorimetric hydrogen ion determination, by diluting it under oil, in a tube of 20 mm. diameter, with 20 c.c. of neutral 0.9 per cent NaCl, containing 7 drops of 0.03 per cent phenol red.

Another 1 c.c. sample of the plasma is transferred to a round flask of about 100 c.c. capacity, 5 c.c. of 0.01 NHCl, which is made up in neutral 0.9 per cent NaCl, are added, and the CO_2 is removed by whirling the mixture vigorously about the flask for at least 1 minute with a rotary motion so that the solution is whirled in a thin layer about the inner wall. The solution is poured into a test tube of the same diameter as that containing the standard, the portion adherent to the walls of the flask being transferred by rinsing with 10 c.c. of 0.9 per cent NaCl divided into three portions. Seven drops of the 0.03 per cent phenol red solution are added and 0.01 N NaOH is run in from a burette, which permits readings to 0.01 c.c., until the color matches that of the standard. As the end point is approached, sufficient 0.9 per cent NaCl is added to bring the volume to 20 c.c.

The 0.01 N NaOH like the 0.01 N NaCl is made up by diluting 1 volume of 0.1 N pure solution to 10 volumes with neutral CO_2-free 1 per cent NaCl.

The use of saline solution instead of water has the advantage of preventing the formation of a permanent cloudy precipitate of globulin.

A peculiar phenomenon occurs as the end point is approached. Each drop appears to change the color past the end point, but within a few seconds the color shifts back and it is seen that at least another drop is needed. Consequently, the final color comparison should not be made until at least 30 seconds after the last

[1] Van Slyke: *Jour. Biol. Chem.*, 52, 495, 1922.

THE RELATIONSHIP OF THE PLASMA BICARBONATE TO ACID EXCRETION, ALKALI TOLERANCE, AND ALVEOLAR CARBON DIOXIDE TENSION*

Condition of subject	Actual bicarbonate reserve	Corresponding results of indirect tests for acidosis					
		24-hour excretion† of $\frac{N}{10}$ acid + NH_3		Carbon dioxide of alveolar air		Sodium bicarbonate required to turn urine alkaline	
	Plasma bicarbonate CO_2 reduced to 0°, 760 mm.	(*a*) C.c. per kg. (*b*) Approximate c.c. per 60 kg. person	Reliability in diabetes	(*a*) Mm. tension. (*b*) Approximate per cent	Reliability in diabetes	(*a*) Gm. per kg.‖ (*b*) Approximate gm. for a 60 kg. person	Reliability in diabetes
	Vol. per cent	C.c.					
Normal resting adult.‡ Extreme limits of bicarbonate reserve.	80–53	(*a*) 0–27 (*b*) 0–1600	Good	(*a*) 53–35 mm. (*b*) 6.8–4.7 per cent	May indicate some acidosis in its absence	(*a*) 0–0.5 (*b*) 0–30	May indicate acidosis in its absence
Mild acidosis, no pronounced symptoms.	53–40	(*a*) 27–65 (*b*) 1600–4000	Good§	(*a*) 35–27 mm. (*b*) 4.7–3.6 per cent	May indicate more acidosis than is present	(*a*) 0.5–0.8 (*b*) 30–50	
Moderate to severe acidosis. Symptoms may be apparent.	40–30	(*a*) 65–100 (*b*) 4000–6000	Liable to considerable error in either direction§	(*a*) 27–20 mm. (*b*) 3.6–2.7 per cent	Good	(*a*) 0.8–1.1 (*b*) 50–65	May indicate much more acidosis than is present
Severe acidosis. Symptoms of acid intoxication.	Below 30	(*a*) Over 100 (*b*) Over 6000		(*a*) Below 20 mm. (*b*) 2.7 per cent	Good	(*a*) Over 1.1 (*b*) Over 65	

*Van Slyke: *Jour. Biol. Chem.*, 33, 271, 1918.

† Measured either in 24-hour urine or on specimen from shorter period calculated to 24-hour basis.

§ After bicarbonate administration likely to indicate more acidosis than is present.

‖ The figures tabulated in this column also indicate the doses of bicarbonate necessary to restore the alkali reserve to normal from acidosis of the severity indicated by the corresponding plasma CO_2 figures in the first column.

‡ Schloss (*Am. Jour. Dis. Chil.*, 13, 218, 1917) finds values of 63–46 in the plasma of normal infants, *i.e.*, about 10 c.c. lower than in adults.

drop of 0.01 N NaOH has been added. It is better to overrun the end point by a drop, rather than stop short of it when in doubt. In calculating the results the c.c. of 0.01 N NaOH used in the titration is subtracted from the c.c. required to neutralize to the same indicator 5 c.c. of the 0.01 N HCl used. The number is approximately 5 but usually varies from it slightly because of difference in factors of acid and alkali and because of the calibration error of the 5 c.c. pipette used in measuring the acid. The maximum accuracy is obtained by performing a preliminary titration on 5 c.c. of the acid plus 15 c.c. of distilled water, using the same pipette, indicator, and end point as in the plasma titration. The titration result represents c.c. of 0.01 M $NaHCO_3$ per c.c. of plasma and it is transformed into terms of molecular concentration of $NaHCO_3$ by merely dividing by 100. If the $NaHCO_3$ molecular concentration is multiplied by 2240 or the number of c.c. of 0.01 N HCl used in the titration by 22.4, the volume per cent of bicarbonate CO_2 in the plasma is obtained and the results can thus be compared with those obtained by the CO_2 method. The standard 0.01 N NaOH must be protected from atmospheric CO_2 and kept in paraffined bottles to prevent solution of alkali from the glass.[1] The burette should be filled with fresh solution each day. The carbonate-free solution is made by dissolving the NaOH in an equal volume of H_2O. On standing the Na_2CO_3 settles to the bottom. 2.75 c.c. of the clear supernatant solution is diluted to 5 L. and standardized by titration with neutral red against 0.01 N HCl. It is preferable to run the acid into the alkali as the color change occurs without the time lag observed when alkali is added to acid.

Interpretation.—The results agree closely with those of the CO_2 capacity method over the range of bicarbonate concentrations (0.03 to 0.01 M) ordinarily encountered in man, even in severe acidosis. Below this range the titration continues to give accurate results, while the CO_2 capacity method gives somewhat higher values. For clinical and most experimental purposes, however, it appears that the two methods give so nearly identical results that they may be used interchangeably.

(c) **Titratable Alkali of Blood.—Method of Greenwald and Lewman.**[2]—**Principle.**—An excess of picric acid is added to the blood. This precipitates the protein In the filtrate, free and total picric acid are determined. The difference represents picric acid combined with the bases of the blood and hence the alkali originally combined with carbonic acid and protein in the blood.

Procedure.—The blood is collected in the usual manner, potassium oxalate being used to prevent coagulation. Excessive amounts of oxalate must not be used. No precautions to protect the blood against loss or gain of oxygen or carbon dioxide

[1] Testing Standard 0.01 N NaOH for Carbonate.—The solutions should be made up using only boiled water, be kept in paraffine-lined bottles, and be protected from atmospheric CO_2 by soda-lime tubes. They should be tested for carbonate as follows:

To 5 c.c. of 0.01 N HCl in a 200 c.c. round flask, add from a freshly filled burette about 4.8 c.c. of the 0.01 N NaOH to be tested, 0.3 c.c. of neutral red solution. The mixture should be strongly acid to the indicator. The solution is rotated for one minute in the flask to permit the escape of CO_2, and is then transferred to a 50 c.c. Erlenmeyer flask and titrated as in plasma analyses, the total amount of 0.01 N NaOH required to give the end point being noted.

A duplicate titration is performed in the same way except that there is no agitation to remove carbon dioxide, the 0.01 N HCl plus 20 c.c. of water being placed directly in the 50 c.c. Erlenmeyer flask, and the 0.01 N NaOH being added with a minimum of stirring.

If there is no carbonate in the standard NaOH solution the two titrations give identical results. The difference should preferably not exceed 0.1 c.c., and if it exceeds 0.2 c.c. the alkali should not be used.

[2] Greenwald and Lewman: *Jour Biol. Chem.*, 54, 263, 1922.

are necessary. Five c.c. of the oxalated blood are measured into a 50 c.c. volumetric flask and are diluted with about 10 c.c. of water. About 30 c.c. of 1 per cent picric acid solution[1] are then added, with shaking, and the mixture is diluted with water to the mark. The mixture may be filtered in 30 minutes, or, if shaken frequently, in 10 minutes or less. Thirty c.c. of the filtrate are heated to boiling in a 150 c.c. Erlenmeyer flask, cooled in water, and then titrated with N/100 NaOH, using 4 drops of 0.01 per cent methyl red as indicator. After the end-point has been reached, 4 drops of 0.01 per cent phenol red are added and a second titration is made to the appearance of a definite orange color. Finally, 8 drops of 1 per cent thymol-phthalein may be added, and a third titration obtained. Daylight or light from a "Daylite lamp" should be used. The liquid is then strongly acidified with acetic acid, heated to boiling, and the picric acid is precipitated by the addition, from a dropping tube, of a 1 per cent solution of nitron in 10 per cent acetic acid. The precipitate is filtered out on a tared Gooch crucible, is washed with water, and is then dried at 110° for 1 hour, cooled, and weighed.

Calculation.—If thymolphthalein has been employed, a correction of 0.1 mg. for each drop of 1 per cent solution used is subtracted to allow for the amount of thymolphthalein carried down with the precipitate. The molecular weight of the nitron picrate is 541. Theoretically, if each molecule of picric acid is neutralized by 1 molecule of NaOH 1 mg. of nitron picrate = 0.1848 c.c. of N/100 NaOH. This is the value actually obtained by experiment with methyl red. As has already been explained, other factors must be used with other indicators. These factors are best obtained by titrating 5 c.c. portions of 1 per cent picric acid with N/100 NaOH, using all three indicators in turn, then precipitating with nitron, weighing the nitron picrate, and calculating its value in terms of N/100 NaOH. The values found by Greenwald and Lewman were 1 mg. of nitron picrate = 0.1890 c.c. of N/100 NaOH to phenol red and 0.1958 c.c. to thymolphthalein.

From the alkali equivalent of the nitron picrate, there is deducted the amount of alkali required to neutralize, to the corresponding indicator, the free acid in the sample. The difference represents the amount of alkali previously combined with protein or with carbonic acid in the blood. The results are then calculated in terms of c.c. of N/10 NaOH per 100 c.c. of blood by dividing by 3 and multiplying by 10. The error is within ±1 per cent with methyl red and is less than ±2 per cent with the other indicators.

3. Alkali Reserve.—Indirect Method. Alveolar Carbon Dioxide Tension. Fridericia's Method.[2]

[1] *Preparation of Reagents. Picric Acid.*—Merck's "Blue Label" picric acid is satisfactory. Dissolve 20 gm. in hot water in a 2,000 c.c. volumetric flask, allow to cool, and dilute to mark.

0.01 *and* 0.005 *N NaOH.*—Prepared daily by dilution of 0.1 N NaOH with CO_2-free water.

Indicators.—Methyl red and phenol red. 0.4 per cent solutions are prepared according to Clark by dissolving 0.1 gm. in 7.4 and 5.7 c.c., respectively, of 0.05 N NaOH and diluting to 25 c.c. For use in this method, these solutions are diluted with 9 volumes of water.

Thymolphthalein.—A 1 per cent solution in alcohol, to which there is added enough NaOH to give a slight blue color.

Nitron.—A filtered 1 per cent solution in 10 per cent acetic acid. *Recovery of excess nitron from the filtrates.* These are evaporated to small volume, cooled, and made strongly alkaline with ammonia. The nitron separates out, is filtered, washed with a little water, air-dried, and is then ready for use.

[2] Fridericia: *Hospitalstidende,* Copenhagen, 57, 585, 1914; Poulton: *Brit. Med. Jour.*, 2, 392, 1915.

Principle.—The method of determination is based upon the absorption, by means of potassium hydroxide, of the carbon dioxide from a known amount of alveolar air. The apparatus is so graduated that the decrease in volume may be read in per cent.

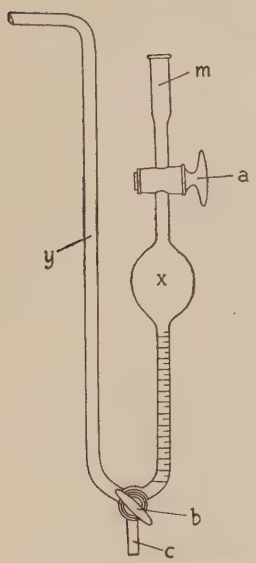


FIG. 134.—FRIDERICIA APPARATUS.

Procedure.—The subject must sit quietly in a chair and breathe naturally,[1] holding the apparatus (shown in Fig. 134) in front of him with the cock *a* open and *b* in a position connecting *x* with *y*. After taking a normal inspiration he places the mouthpiece *m* into his mouth and blows as hard and as quickly as possible through the apparatus thus washing it out and leaving it filled with alveolar air. The cock *a* is at once closed and the whole apparatus is immersed in water for 5 minutes. By this means the alveolar air in *x* and *y* is cooled to a temperature which remains constant throughout the experiment, and the contraction in volume causes the alveolar air in the lower part of *y* to be drawn back into *x*.[2] At the end of 5 minutes the cock *b* is turned so as to connect *y* with *c*, thus closing *x* which then contains exactly 100 c.c. of alveolar air at atmospheric pressure and at the temperature of the water in which the apparatus is immersed, this temperature remaining constant throughout the determination. The apparatus is removed from the water, the tube *c* is placed beneath the surface of some 10 per cent sodium hydroxide solution, some of the alkali is drawn up into *y*, the apparatus is held in such a position that *y* is rather depressed in order to prevent the escape of small bubbles of gas from *x*, the cock is turned so as to connect *y* with *x*, and some of the alkali is forced into *x*. The cock *b* is at once turned, closing *x* and connecting *y* with *c* through which the remainder of the alkali is allowed to flow. The apparatus is inverted several times during the course of half a minute which is sufficient time for the absorption of all the carbon dioxide. It is then returned to the water which rises through *c* into *y*, after which *b* is turned to connect *y* and *x* and the whole is allowed to remain for 5 minutes to again equalize the temperature. It is then raised rapidly until the water in the graduated portion of *x* is at the same level as the water outside the apparatus, i.e., when the gas within the tube *x* is under atmospheric pressure.

Calculation.—The reading of the bottom of the meniscus of the fluid in *x* is taken and represents, without any further calculation or correction, the percentage of carbon dioxide in the alveolar air.

[1] It is especially important to caution the subject against the very natural inclination to take an abnormally deep inspiration just before blowing through the apparatus, and also to see that, in seeking to avoid this fault, the breath is not held just before the sample is taken.

[2] Any diffusion with the outside air at the top of *y* will not reach to the bottom of the tube owing to its length.

Interpretation.—The sample of air obtained by this method (if properly taken) represents more nearly air whose CO_2 tension is the same as that of arterial blood, *i.e.*, true alveolar air, than does air obtained by "rebreathing." The results obtained by this method, then, are from 5 to 10 per cent lower than those obtained by the Marriott method below. The average normal value for men[1] is about 5.5 volumes per cent of carbon dioxide. In women and children the normal value is somewhat lower. In acidosis the carbon dioxide falls and in diabetic coma may go as low as 1 or 2 per cent. A value of 2 per cent means that coma may supervene within 24 hours. A value of 3 per cent or 4 per cent is less dangerous; in the worst event coma will not come on for at least two or three days. For detailed data as to alveolar carbon dioxide tension under different conditions see table on page 439.

4. Alkali Reserve. Indirect Method Alveolar Carbon Dioxide Tension Marriott's Method.[2]—While this method is open to criticism because of the liability of error in the collection of the sample and, more fundamentally, because of various factors (psychical, etc.) other than acidosis which may influence the carbon dioxide tension, nevertheless, it is of considerable value and has been rather widely adopted for clinical use.

Principle.—By rebreathing air under certain definite conditions a sample is obtained whose carbon dioxide tension is virtually that of venous blood. The method of analysis of this sample depends on the fact that if a current of air containing carbon dioxide is passed through a solution of sodium carbonate or bicarbonate until the solution is saturated, the final solution will contain sodium bicarbonate and dissolved carbon dioxide. The reaction of such a solution will depend on the relative amounts of the alkaline bicarbonate and the acid carbon dioxide present. This, in turn, will depend on the tension of carbon dioxide in the air with which the mixture has been saturated and will be independent of the volume of air blown through, provided saturation has once been attained. High tensions of carbon dioxide change the reaction of the solution toward the acid side. Low tensions have the reverse effect; hence the reaction of such a solution is a measure of the tension of carbon dioxide in the air with which it has been saturated. A suitable indicator is added to the solution and its reaction (after the passage of the alveolar air) is determined by comparison with a set of suitable standards.

Apparatus.—The complete apparatus, including rubber bag for collection of sample, standardized phosphate mixture sealed in tubes, the standard bicarbonate solution, tubes, color comparison box and other accessories may be obtained from Hynson, Westcott and Dunning, Baltimore, Maryland.

Procedure.—Collection of the alveolar air. The method of collection is essentially that of Plesch,[3] as modified by Higgins.[4] A rubber bag of approximately

[1] Beddard, Pembrey, and Spriggs: *Brit. Med. Jour.* 2, 389, 1915.
[2] Marriott: *Jour. Am. Med. Ass'n*, 66, 1594, 1916.
[3] Plesch: *Ztschr. f. exper. Path. u. Therap.*, 380, vi, 1909.
[4] Higgins: *Pub.* 203, *Carnegie Institution of Washington*, 1915, p. 168.; Boothby, W. M., and Peabody, F. W.: A comparsion of Methods of Obtaining Alveolar Air, *Arch. Int. Med.*, March, 1914, p. 497.

1500 c.c. capacity[1] is connected by means of a short rubber tube to a glass mouthpiece.[2] About 600 c.c. of air are blown into the bag with an atomizer bulb, and the rubber tube clamped off by a pinchcock. The subject should be at rest and breathing naturally.[3] At the end of a normal expiration, the subject takes the tube in his mouth; the pinchcock is released and the subject's nose closed by the observer. The subject breathes back and forth from the bag 4 times in 20 seconds, emptying the bag at each inspiration. The observer should indicate when to breathe in and out. Breathing more frequently will not greatly alter the results. At the end 20 seconds, the tube is clamped off and the air analyzed. The analysis should be carried out within 3 minutes' time, as carbon dioxide rapidly escapes through rubber.

The foregoing precedure applies to patients who are capable of coöperating to some extent. In the case of comatose patients, the initial amount of air in the rubber bag must be greater (1000 c.c. at least), and the period of rebreathing prolonged to 30 seconds.[4] This is necessary, as it is not feasible that the bag be completely emptied of air at each inspiration; and therefore a longer time is required for the carbon dioxide tension in the bag and in the lungs to become equal. The initial amount of air in the bag should be such that it is at least one-half and preferably as much as two-thirds emptied at each inspiration. Since comatose patients cannot hold the mouthpiece, some form of mask is necessary. This may be a gas anesthetic mask[5] or such a device as described below for use with infants.

A special mask has been devised for the collection of alveolar air from infants.[6] It is made from the nipple of a wide-mouth (Hygeia) nursing bottle and a piece of thin rubber tissue (dental dam). A sheet of the tissue (8 by 10 inches) is perforated in the center by a piece of hot metal or glass tubing of large bore. The hole is stretched and pulled over the nipple and slipped down to the lower rim. A small amount of rubber cement is applied to hold the tisssue and nipple together. A strip of adhesive plaster $\frac{3}{8}$ inch wide is applied around the rim of the nipple so as to overlap the rubber tissue and hold it firmly in place. The extreme tip of the nipple is cut off and a short glass tube, $\frac{3}{8}$ inch in diameter, inserted.

"In making a collection of alveolar air from infants a rubber bag of 500 c.c. capacity is connected with the mask and partially filled with air by means of an aspirator bulb. The neck of the bag is closed off by a pinchcock or with the fingers, the mask placed over the nose and mouth of the infant and the rubber tissue closely drawn around the face so as to prevent the escape of air. The mask should, if possible, be placed over the face just at the end of expiration. Respirations are allowed to continue for from 28 to 32 seconds, and at the end of an expiration

[1] A basket ball bladder or a hot water bag answers very well. If the latter is used, the neck may be closed by a rubber stopper carrying a short glass tube $\frac{3}{8}$ inch in internal diameter.

[2] An ordinary piece of glass tubing with rounded edges, $1\frac{1}{2}$ inch long and $\frac{3}{8}$ inch in internal diameter.

[3] Especially to be guarded against is a deep, voluntary inspiration just before the collection begins, as this causes too low a determination.

[4] The bag is clamped at the end of that expiration occurring nearest to 30 seconds, as no great error is introduced by prolonging the time of rebreathing by 2 or 3 seconds.

[5] Some advantage may be gained by inserting a large three-way stopcock of metal or glass between the mask and the bag. When such a stopcock is used, the mask is first put in place on the patient's face with the cock so turned that he breathes the outside air for a few respirations. At the end of an expirioatn the cock is quickly turned so as to bring the bag and the mask into connection.

[6] Howland and Marriott: *Am. Jour. Dis. Child.*, May, 1916.

the neck of the bag is closed off and the mask removed from the face. We have found that it is necessary that the infant should be breathing quietly for 1 minute previous to the collection of the air sample, as vigorous crying, just before the mask is put on, leads to a lowering of the carbon dioxide tension, as determined, by several millimeters. Crying during the collection of the sample almost invariably occurs and facilitates mixing of the gases. The effect is to raise the tension somewhat if crying is very vigorous, but not to such an extent as to be significant. The initial amount of air in the bag must be such that during inspiration the bag is from one-half to two-thirds empty, but never completely collapsed. The amount of air required for infants under 1 year of age varies from 250 c.c. to 400 c.c."

The mask described above for use with infants may be very conveniently used for the collection of alveolar air samples from dogs. The animal's nose is inserted into the mask and the rubber tissue drawn closely around the muzzle. The time of a collection need not exceed 25 seconds.

Analysis of Sample.—In analyzing a sample of air, about 2 or 3 c.c. of the standard bicarbonate solution are poured into a clean test-tube of the same diameter as the tubes containing standard phosphate solutions, but from 100 to 150 mm. long. Air from the bag is then blown through the solution by means of a glass tube drawn out to a fine capillary point, until the solution is saturated, as shown by the fact that no further color change occurs.[1] The tube is stoppered and the color immediately compared with that in the standard tubes. By interpolation, one can readily read to millimeters. Color changes are not quite so sharp above 35 mm. as at the lower end of the scale, but here changes are of less significance. In making the color comparisons the solution being compared is placed between the two standards which it most nearly matches. When there is doubt as to whether the color of the solution is higher or lower than one of the standards, changing the order in which the tubes are placed in the comparison box will generally make the relationship clear.

The standard solutions described are so prepared as to give correct results when the determination is carried out at a temperature of from 20 to 25°C. (from 68° to 77°F.). When the room temperature is considerably higher or lower than these points it is advisable to immerse the tubes in water at approximately 25°C. during the blowing. They may be removed from the water for the color comparison, however, provided this is quickly made. The differences due to ranges of temperature occurring under ordinary circumstances are practically negligible.[2]

Calculation.—The standard tubes are marked to indicate the carbon dioxide tension in millimeters of mercury, and the readings can be estimated to about 2 mm.

Interpretation.—In normal adults at rest the carbon dioxide tension in the alveolar air, determined and described above, varies from 40 to 45 mm. Tensions between 30 and 35 mm. are indicative of a mild degree of acidosis. When the tension is as low as 20 mm., the individual may be considered in imminent danger. In coma, associated with acidosis, the tension may be as low as 8 or 10 mm. In infants, the tension of carbon dioxide is from 3 to 5 mm. lower than in adults.

[1] If the operator first blows his own breath through the solution so as to bring it into approximate equilibrium with alveolar air, saturation may be accomplished with as little as 100 c.c. of air from the bag, blown through during 30 seconds. The same bicarbonate solutions may be used for repeated determinations.

[2] No correction for barometric pressure is required as from the nature of the determination, barometric fluctuations are self-corrective. Variations in the temperature of the subject are never great enough to affect the value as much as 1 mm. and therefore may be neglected.

Conditions other than acidosis may affect the carbon dioxide tension. Stimulation of the respiratory center leads to increased pulmonary ventilation and a consequent lowering of the alveolar carbon dioxide tension. Such stimulation may be brought about by caffein[1] and possibly also by intracranial lesions. The respiratory center may be depressed by morphin, and as the result of certain[1] infections. This leads to an increased carbon dioxide tension. Changes in the excitability of the respiratory center, however, are but rarely great enough to affect significantly the composition of the alveolar air.

"Alveolar" air collected as described above is essentially air which has come in equilibrium with the venous blood in the pulmonary capillaries. The tension of carbon dioxide is approximately that in the venous blood. "Alveolar" air collected by the Haldane or Fridericia methods is air which has come in approximate equilibrium with the arterial blood, and hence is of a carbon dioxide tension from 10 to 20 per cent lower.

Changes in the pulmonary epithelium such as would prevent the air in the lungs from coming in equilibrium with the blood in the capillaries would, of necessity, affect the composition of the alveolar air. Since very little is known as yet regarding the exact effect of such changes, one is hardly justified in drawing conclusions regarding acidosis from the composition of the alveolar air in patients with pulmonary affections.[2]

5. Alkali Reserve. *Indirect Method. Index of Acid Excretion in Urine. Method of Fitz and Van Slyke.*[3] *Principle.*—The method depends upon the determination of the rate of excretion of acid (NH_3 + titratable acid) from which the plasma carbon dioxide capacity is calculated.

Procedure.—Collect the urine for 24 hours (or if desired for a period of 1 or 2 hours during which the subject ingests neither food nor water). In the latter case the urine collection should not be too soon after a meal. Measure, carefully, the volume of the urine and determine its ammonia content according to the method given on page 724 and the titratable acid according to the method given on page 705. Obtain the body weight of the patient.

Calculation.—The plasma bicarbonate may be calculated by substitution in the following equation.

$$\text{Plasma Carbon Dioxide Capacity}^4 = 80 - 5\sqrt{\frac{D}{W}}$$

D = Rate of excretion per 24 hours.
W = Body weight in kilograms.

The value D is equal to the product VC, in which V is the 24 hour volume[5] expressed in liters, and C the sum of the ammonia (expressed as c.c. of N/10

[1] Higgins and Means: *Jour. Pharmacol. and Exper. Therap.*, 1915, vii, 1.
[2] Means, Newburgh and Porter: *Boston Med. and Surg. Jour.*, 1915, clxxiii, 742.
[3] Fitz and Van Slyke: *Jour. Biol. Chem.*, 30, 389, 1917; Van Slyke: *Ibid.*, 33, 271, 1198; Barnett: *Jour. Biol. Chem.*, 33, 267, 1918.
[4] The value 80 represents the maximum normal value of plasma bicarbonate. Under such a condition the titratable acid and ammonia excretion tend to approach zero.
[5] If the urine is collected for only 1 or 2 hours its volume is, of course, multiplied by 24 or 12 as the case may be.

NH_3 per liter of urine) plus the titratable acid (expressed as c.c. of N/10 acid per liter of urine). For practical purposes the acid excretion may, without going through the calculation of the formula, be interpreted directly into terms of clinical severity of acidosis, as indicated in the table on page 439, e.g., an excretion exceeding 27 c.c. of N/10 ammonia plus titratable acid per kilo indicates acidosis, which usually becomes critical in severity if the excretion approaches 100 c.c. per kilo.

Interpretation.—After careful investigation in which the relationship between the carbon dioxide capacity of plasma and the excretion rate and concentration of total urinary acid excreted in excess of mineral bases was determined, Fitz and Van Slyke concluded that no other equation including excretion rate and concentration was so satisfactory as the above simplification of that used by Ambard for urea and chloride.[1]

The value $80 - 5\sqrt{\frac{D}{W}}$ indicates, with an error which is usually less than 10 volumes per cent, the level of the plasma carbon dioxide capacity. Diabetics receiving bicarbonate administrations are exceptions, the blood bicarbonate in such cases being, as a rule, much higher than indicated by the urine.

Of the two indirect measures of alkali reserve the alveolar carbon dioxide determination appears the more accurate in measuring the more severe stages of diabetic acidosis, such as are encountered in threatened coma, while the index of acid excretion is the more accurate in measuring the more common intermediate stages.[2]

In nephritis, acidosis (lowered blood bicarbonate) may occur without increase in acid excretion or even with decrease of the latter. Consequently the excretion cannot be used as an indicator of acidosis when nephritis is present.

For values of the acid index under different conditions see table, page 439.

6. Alkali Tolerance.[3]—This method is quite reliable for proving the absence of acidosis, but is not particularly dependable for showing either the presence or the degree of acidosis when it exists. This seems to be due in part at least to the fact that in conditions associated with acidosis the power of the kidney for excretion of alkalies may be markedly impaired.

Principle.—Sodium bicarbonate is administered in small amounts, either by mouth or intravenously until the reaction of the urine changes from acid to alkaline. The amount of bicarbonate is then noted.

[1] Ambard: *Physiologie normale et pathologique des reins*, Paris, 1914.
[2] Stillman, Van Slyke, Cullen, and Fitz: *Jour. Biol. Chem.*, 30, 405, 1017.
[3] Sellards: *Bull. Johns Hopkins Hosp.*, 23, 289, 1912; Palmer and Henderson: *Arch. Int. Med.*, 12, 153, 1913; Palmer and Van Slyke: *Jour. Biol. Chem.*, 32, 499, 1917.

Procedure.—Give (by mouth) 5 grams of sodium bicarbonate in 100 c.c. of water to the subject under examination. Repeat every half hour until the total bicarbonate administration is equivalent to 0.5 gram per kilogram of body weight unless the urine becomes alkaline before that time. In case the urine does not become alkaline with the above bicarbonate ingestion, continue the administration of the alkali until the urine shows an alkaline reaction.[1] The urine should be voided by the subject before each administration of bicarbonate. Test each specimen of urine with litmus, boiling those samples which are only faintly acid so that any bicarbonate present will be converted to carbonate. Note the number of grams of bicarbonate necessary to produce an alkaline urine.

Interpretation.—Normally the administration of from 5 to 10 grams of bicarbonate is generally sufficient to produce an alkaline reaction in the urine,[2] while in patients suffering from acidosis a greater amount is required. In general a maximum ingestion of 0.5 gram of bicarbonate per kilogram body weight will produce an alkaline urine in a normal person. In mild acidosis this value may be increased to a maximum of 0.8 gram, whereas, moderate acidosis may show a value of 1.1 grams. In severe acidosis with symptoms of acid intoxication the bicarbonate value may exceed 1.1 grams per kilogram body weight. If an alkaline urine is obtained after the administration of 0.5 gram or less of bicarbonate per kilogram body weight one is safe in saying that no acidosis exists. When higher values are obtained, however, they should be confirmed by blood analysis before being accepted. For data as to alkali tolerance under different conditions see table on page 439.

7. Hydrogen Ion Concentration of Blood Plasma.—*Colorimetric Method of Cullen*[3] *as Modified by Hawkins.*[4]—*Principle.*—Blood is collected with precautions against loss of CO_2. The plasma is diluted with saline solution containing phenol red and the color obtained compared with that of standard phosphate mixtures of known hydrogen ion concentration.

Procedure.—Prepare two test tubes of the same diameter as are used to contain the standards (see below), one containing 5 c.c. of 0.9 per cent sodium chloride solution and the other 5 c.c. of saline indicator solution[5] each covered with a 1 cm. layer of neutral mineral oil. Connect a Luer needle adapter by means of a short piece of rubber tubing, to a 1 c.c. Mohr pipette. Without stasis insert the needle into the subject's vein, attach the adapter and allow the

[1] Because of the likelihood of producing a condition of alkalosis it is advisable not to continue the administration of bicarbonate without evidence from blood analysis showing an alkali deficit. In certain connections alkalosis may be as dangerous for the organism as acidosis (Grant: *Arch. Int. Med.*, 30, 355, 1922).

[2] Henderson and Palmer: *Jour. Biol. Chem.*, 14, 81, 1913.

[3] Cullen. *Jour. Biol. Chem.*, 52, 501, 1922.

[4] Hawkins: *Jour. Biol. Chem.*, 57, 493, 1923.

[5] Prepare fresh for each determination. Add 2.1 c.c. of 0.03 per cent phenol red solution to 100 c.c. of 0.9 per cent sodium chloride solution. Adjust to approximately pH 7.4 by stirring with a fine glass rod dipped into 0.02 N sodium hydroxide.

blood to fill the pipette past the zero mark. (A tourniquet may be used to aid in finding the vein, but the blood should flow into another tube a few seconds after the release of the tourniquet, so that stasis is absent when the adapter is inserted into the needle.) Withdraw the needle from the vein and at once disconnect the rubber tubing from the pipette. Wipe the tip and introduce into each tube, under the oil, 0.4 c.c. of the blood. Stir the contents carefully with a fine glass rod.

Determination.—Centrifuge both tubes at about 1500 r.p.m. for 5 minutes. Compare with standards[1] in a comparator block, estimating between two standards, if necessary. The tubes are placed in the following relative positions:

[1] Reagents and apparatus required:

Standards.—Sørensen's phosphate standards are prepared from Merck's special reagents in steps of pH 0.05 from about pH 7.2 to 7.7. The M/15 phosphate solution should be prepared from special reagent salts (Merck's are satisfactory) by dissolving the following quantities to a liter with distilled water:

$Na_2HPO_4.2H_2O$ "Sørensen salt" ..... 11.87 gm.
or Na_2HPO_4 anhydrous (Merck) ..... 9.47 gm
KH_2PO_4 ..... 9.08 gm.

The proportions of acid and alkaline phosphates are given in the table. These mixtures may be kept for some weeks in Pyrex glass in the refrigerator.

PHOSPHATE MIXTURES
(Phenol Red Range)

pH	M/15 Na_2HPO_4	M/15 KH_2PO_4	pH	M/15 Na_2HPO_4	M/15 KH_2PO_4
	c.c.	c.c.		c.c.	c.c.
7.0	61.1	38.9	7.40	80.8	19.2
7.05	63.9	36.1	7.45	82.5	17.5
7.10	66.6	33.4	7.50	84.1	15.9
7.15	69.2	30.8	7.55	85.7	14.3
7.20	72.0	28.0	7.60	87.0	13.0
7.25	74.4	25.6	7.65	88.2	11.8
7.30	76.8	23.2	7.70	89.4	10.6
7.35	78.9	21.1	7.75	90.5	9.5
.....	.....	.....	7.80	91.5	8.5

Indicator Solution.—The color standards are prepared by adding 0.3 c.c. of 0.03 per cent phenol red (phenolsulphonephthalein) to 15 c.c. of each of the standard buffer solutions. The concentration of dye required varies somewhat with different lots. It is best to prepare a concentrated stock solution, and determine by experiment the dilution required to give satisfactory depth of color over the desired pH range. The indicator solution must be neutral. After the addition of 1 drop to 3 c.c. of redistilled water, the water must not be red.

The color standards must be renewed or checked against a fresh tube of dye at least once every week; as there is a slow fading of color.

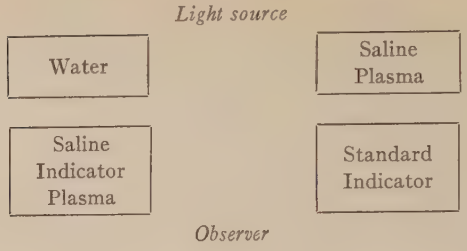
Apparatus.—The comparator required is conveniently made from a block 3 × 6 inches. The holes for the tubes are of 1 inch diameter. The slits for the light are best made by boring two ½ inch holes and gouging out the remaining wood with a chisel. The tube must be of clear, non-alkaline glass of *uniform* diameter. Tubes 20 mm. in diameter are convenient.

Light.—Either daylight or "Daylite" lamps are satisfactory.

Test for Neutrality.—The redistilled water is usually about pH 6.2 to 6.5. The easiest test is that of using both phenol red and methyl red. The water should give no (red) color with either indicator.

The syringe, pipettes, and tubes should be rinsed with redistilled water and dried. Syringes, tubes, and pipettes, washed and sterilized in the usual manner prevalent in bacteriological laboratories, are often dried from alkaline water.

The saline solution must be adjusted to pH 7.4 as described above. The oxalate, when dissolved in water to a 0.5 per cent solution, should not be more alkaline than pH 7.2 to 7.4. The oil is tested by shaking with water containing phenol red and methyl red. The water must remain neutral.



The object of the saline plasma tube is to compensate the standard for the slight color and turbidity of the plasma. The temperature of the saline-indicator-plasma is obtained. It is desirable to make determinations at 20°C., by placing the plasma tubes, together with the necessary standard tubes in a large beaker of water at 20°.

Calculation.—The pH of the blood (human) at 38°C. is obtained from the following equation:

$$pH_{38^\circ} = \text{Colorimetric } pH_{t^\circ} + 0.01\ (t^\circ - 20) - 0.23,$$

in which t° represents the observed temperature. It the tubes are adjusted to 20°, the middle factor naturally drops out.

The empirical correction, − 0.23, ("Cullen correction") is applied to adjust the colorimetric pH values to those determined electrometrically at 38°, and compensates for the protein and salt errors in the colorimetric determination. The correction varies with the species of animal, but unfortunately is not as constant in each species as could be desired.

Hastings and Sendroy[1] claim that the Cullen correction is unnecessary when the readings are made with the tubes at body temperature. They propose a series of bicolor standards consisting of the various ratios of alkaline and acid forms of indicator in pairs of tubes corresponding to the pH at 20° or 38°.

Myers[2] has devised a multiple wedge colorimeter[3] (Fig. 112) for bicolorimetric measurements, using one wedge for the acid form of the indicator, another for the alkaline form and a third for a compensating solution. A technic for the determination of the pH of blood, adapted to the bicolorimeter, has been described.[4] Austin, Stadie and Robinson[5] have shown, however, that under pathological conditions in man, and in the dog, there is considerable variation in the Cullen correction, and Hastings and Sendroy's procedure does not eliminate it. For these cases, they state, corrections must be determined on each serum if colorimetric readings are to be relied upon. A method is described for this purpose.

Interpretation.—The normal pH range of blood is between 7.30 and 7.50. With more refined methods it is probable that this will narrow down to perhaps 7.35 to 7.45. The extreme limits of pathological variation which have been observed are 6.95 to 7.80. For the significance of abnormal pH values see table on page 429.

[1] Hastings and Sendroy: *Jour. Biol. Chem.*, 61, 695, 1924.
[2] Myers: *Jour. Biol. Chem.*, 54, 675, 1922.
[3] Manufactured by E. Leitz, Inc., New York City.
[4] Myers, Schmitz and Booher: *Jour. Biol. Chem.*, 57, 209, 1923.
[5] Austin, Stadie and Robinson: *Jour. Biol. Chem.*, 66, 505, 1925.

8. Hydrogen Ion Concentration—Electrometric Titration Method of Meeker and Oser.[1] **Principle.**—The electrode potential in a half-cell containing diluted blood, protected from air, is balanced by titrating phosphate buffer solutions into a similar half-cell opposing the other in the circuit. The proportions of the buffer solutions required to produce equilibrium, determine the pH of the blood. By using the quinhydrone electrode system bubbling of hydrogen and the consequent loss of CO_2, are obviated. (See Chapter I.)

Procedure.—Blood is drawn directly from the vein into an oxalated tube, under oil to prevent escape of CO_2. Into a clean 30 c.c. beaker introduce 10 c.c. of physiological saline solution and add, from the tip of a spatula, an excess of quinhydrone. Stir vigorously, add a layer of neutral mineral oil and immerse the beaker in a water bath at 38°C. Into this cell suspend a thermometer and a blank gold electrode which is connected to the rest of the circuit according to the diagram Fig. 9. When the temperature reaches 38° add to the other 30 c.c. beaker (titration cell) 10 c.c. of M/15 Na_2HPO_4 (see p. 449) and an excess of quinhydrone, and then immerse the other gold electrode into it. Without delay, introduce 0.8 c.c. of the oxalated blood from a Mohr pipette into the saline under oil and stir thoroughly for 20 to 30 seconds. Connect the solutions in the two half-cells by means of a salt bridge of saturated KCl (which may be made into a gel by introducing 2 per cent agar) and immediately titrate M/15 KH_2PO_4 (p. 449) from a microburette into the titration cell. Tap the key at intervals, more frequently as the extent of deflection of the galvanometer is diminished. At zero deflection read the burette.

Calculation.—From the proportions of the two phosphate solutions, the pH of the blood at 38°C. may be obtained from the equation:

$$pH = 6.81 + \log \frac{\text{c.c. } Na_2HPO_4}{\text{c.c. } KH_2PO_4}$$

The curve for this equation may be constructed more simply from the data given on page 449. (See also Fig. 8.) One may then read directly the pH from the proportion of one of the buffers in the final mixture.

9. Acetone Bodies.—For methods of determining acetone, acetoacetic and β-hydroxybutyric acids in the blood see: Van Slyke and Fitz, *Jour. Biol. Chem.* 32, 495, 1917; Marriott: *Jour. Biol. Chem.* 16, 289, 293, and 295, 1913.

10. Determination of Oxygen and Oxygen Capacity (or Hemoglobin) of Blood.—*Volumetric Methods.*—It is possible to determine the oxygen of the blood using the same apparatus as that employed for the CO_2 estimation (see p. 432) suitable precautions being taken in collecting the blood for analysis. The *oxygen content* of blood represents the total volume of oxygen, both physically dissolved and combined with hemoglobin, present in 100 c.c. of blood in the condition in which it flows through the veins or arteries. For the determination of oxygen content it is, therefore, necessary to collect the blood so as to avoid the oxygenating effect of air, *i.e.*, under oil. An aspirating tube such as illustrated in Fig. 131 serves this purpose.

[1] Meeker and Oser: *Jour. Biol. Chem.*, 67, 307, 1926.

The *oxygen combined with hemoglobin* in arterial or venous blood differs from the oxygen content in that a correction is made for free, physically dissolved oxygen. The same precautions in the collection of the sample must, of course, be taken.

The *oxygen capacity* of blood represents the volume of oxygen required to combine with all of its hemoglobin (oxyhemoglobin and reduced hemoglobin). It, therefore, constitutes a measure of the latter since it is established that one gram of hemoglobin combines with 1.34 c.c. of oxygen, or in other words each c.c. of oxygen capacity represents 0.746 gm. of hemoglobin. Blood for this determination may be collected without oil inasmuch as aeration (oxygenation) is the first step in the procedure.

The *oxygen unsaturation* of venous or arterial blood is a measure of the *absolute* concentration of reduced hemoglobin and is obtained by subtracting from the *oxygen capacity* (total hemoglobin), the *oxygen combined with hemoglobin* as defined above. The distinction between this and the relative unsaturation, which is the *per cent* of total hemoglobin in the reduced state, should be kept in mind. The use of the absolute rather than the relative expression is due to the fact that cyanosis is regarded as the result of an increased absolute concentration of the bluish reduced hemoglobin.

Determination of Oxygen Capacity.—Stir the special oxygen reagent (see p. 466 to obtain emulsification of the caprylic alcohol. Introduce 7.5 c.c. into cup *b* (Fig. 130) and de-aerate by shaking in the evacuated extraction chamber. Mix the blood thoroughly with a stirring rod and transfer to a 250 c.c. separatory funnel in which it is completely aerated by rotating in a tin layer over the interior surface of the funnel. (For oxygen determinations other than capacity, simply mix the blood under the oil and omit aeration.) Force 6 c.c. of the gas-free reagent into the cup of the apparatus. By means of a differential pipette (graduated to deliver between marks, Fig. 139) transfer exactly 2 c.c. of the blood directly into the extraction chamber by keeping the tip of the pipette immersed to the bottom of the reagent in the cup and regulating the admission by the finger or stop-cock on the pipette and cock *e* of the apparatus. Never allow more than a few millimeters of blood to accumulate in the cup. An Ostwald transfer pipette may be used for this purpose, the final drop of blood being forced out of the immersed tip by expanding the air in the pipette with the heat of the hand, keeping the mouth of the pipette closed. Admit the remaining blood and all but 1 c.c. of the reagent from cup *b* into the extraction chamber. With a medicine dropper discard the excess reagent and introduce a few drops of mercury as a seal. Evacuate and shake until the volume of extracted gas ($O_2 + CO_2 + N_2$) is constant. This should be determined by measuring the gas at atmospheric pressure, and will be found to consume from 5 to 10 minutes. Create a slight negative pressure within the pipette by bringing the level of solution to the 2 c.c. graduation. Rinse cup *b* with distilled water and introduce 0.5 c.c. of a 2 per cent NaOH solution previously aerated. By means of the negative pressure within

TABLE OF FACTORS FOR CALCULATION.

Temperature	$f = \frac{B - w}{760(1 + 0.00367\,t)}$ factor by which gas measured moist at $t°$, B mm., is reduced to 0°, 760 mm.[1]	α'_{CO_2}	Air,[2] measured at room temperature and pressure, dissolved by		$1.017f\left(1 + \frac{S}{50 - S}\alpha'_{CO_2}\right)$ factor by which the volume of CO_2 obtained after 1 extraction is multiplied in order to obtain the volume of CO_2 reduced to 0°, 760 mm., contained in the solution analyzed	
			2.5 c.c. H_2O	5 c.c. H_2O	S = 2.5 c.c.	S = 5.0 c.c.
°C.			*c.c.*	*c.c.*		
15	$0.932 \times \frac{B}{760}$	1.075	0.052	0.105	$1.002 \times \frac{B}{760}$	$1.061 \times \frac{B}{760}$
16	$0.928 \times \frac{B}{760}$	1.043	0.051	0.101	$0.995 \times \frac{B}{760}$	$1.053 \times \frac{B}{760}$
17	$0.924 \times \frac{B}{760}$	1.015	0.050	0.100	$0.989 \times \frac{B}{760}$	$1.046 \times \frac{B}{760}$
18	$0.919 \times \frac{B}{760}$	0.989	0.049	0.098	$0.983 \times \frac{B}{760}$	$1.038 \times \frac{B}{760}$
19	$0.915 \times \frac{B}{760}$	0.966	0.048	0.096	$0.978 \times \frac{B}{760}$	$1.030 \times \frac{B}{760}$
20	$0.910 \times \frac{B}{760}$	0.942	0.047	0.095	$0.972 \times \frac{B}{760}$	$1.022 \times \frac{B}{760}$
21	$0.906 \times \frac{B}{760}$	0.919	0.046	0.093	$0.966 \times \frac{B}{760}$	$1.015 \times \frac{B}{760}$
22	$0.901 \times \frac{B}{760}$	0.896	0.045	0.091	$0.960 \times \frac{B}{760}$	$1.008 \times \frac{B}{760}$
23	$0.897 \times \frac{B}{760}$	0.873	0.045	0.090	$0.954 \times \frac{B}{760}$	$1.001 \times \frac{B}{760}$
24	$0.892 \times \frac{B}{760}$	0.850	0.044	0.088	$0.948 \times \frac{B}{760}$	$0.993 \times \frac{B}{760}$
25	$0.888 \times \frac{B}{760}$	0.828	0.043	0.086	$0.942 \times \frac{B}{760}$	$0.986 \times \frac{B}{760}$
26	$0.883 \times \frac{B}{760}$	0.808	0.042	0.084	$0.936 \times \frac{B}{760}$	$0.978 \times \frac{B}{760}$
27	$0.878 \times \frac{B}{760}$	0.789	0.041	0.083	$0.931 \times \frac{B}{760}$	$0.971 \times \frac{B}{760}$
28	$0.873 \times \frac{B}{760}$	0.772	0.040	0.081	$0.924 \times \frac{B}{760}$	$0.964 \times \frac{B}{760}$
29	$0.868 \times \frac{B}{760}$	0.755	0.040	0.080	$0.918 \times \frac{B}{760}$	$0.957 \times \frac{B}{760}$
30	$0.863 \times \frac{B}{760}$	0.738	0.039	0.078	$0.912 \times \frac{B}{760}$	$0.950 \times \frac{B}{760}$

[1] To calculate O_2 or hemoglobin when $O_2 + N_2$ volume is measured, multiply gas volume by f, to reduce to 0°, 760 mm., and by such factor as is necessary (100 when 1 c.c. of blood is used, 50 when 2 c.c. are used) to bring results to volume per cent basis. Then for

a. O_2 content, subtract ..... 1.36 vol. per cent N_2

b. O_2 bound by hemoglobin in venous blood, subtract ..... 1.5 vol. per cent N_2 + dissolved O_2

c. O_2 bound by hemoglobin in arterial blood, subtract ..... 1.7 vol. per cent N_2 + dissolved O_2

d. O_2 bound by hemoglobin in blood saturated with air at 20°, subtract ..... 2.1 vol. per cent N_2 + dissolved O_2

Per cent of normal hemoglobin (Haldane scale) $= \frac{100d}{18.5} = 5.41\,d$.

Grams of hemoglobin per 100 c.c. of blood $= 0.746\,d$.

Per cent of total hemoglobin saturated with $O_2 = \frac{100b}{d}$ or $\frac{100c}{d}$.

Volumes per cent O_2 unsaturation $= d - c$ or $d - b$.

[2] The dissolved air is given as measured at room temperature. It is subtracted from the air + CO_2 volume, measured after one extraction of plasma or aqueous carbonate solution, in order to obtain the CO_2, which is then multiplied by $1.017f\left(1 + \frac{S}{50 - S}\alpha'_{CO_2}\right)$ in order to obtain the total volumes per cent of CO_2 in the solution analyzed. When whole blood is analyzed, the air correction cannot be used, because of the O_2 present, and the CO_2 must be determined by absorption with NaOH solution. The volume of gas absorbed is then multiplied by the above factor.

The factor 1.017, being empirical, may vary slightly for different apparatus.

the pipette, admit this solution slowly, followed by a thin stream of mercury, which serves to break the column of NaOH which usually forms in the capillary of the pipette. Allow one minute for drainage and bring the gas ($O_2 + N_2$) to atmospheric pressure as described in the determination of CO_2. Read the volume of gas, the temperature and the barometric pressure.

Calculations:

V = observed volume of gas ($O_2 + N_2$).
t = temperature in °C.
B = barometric pressure in mm. Hg.
w = tension of aqueous vapor (see Appendix).

$$\text{Volume per cent } O_2 \text{ capacity} = \left(\frac{(B - w)}{760(1 + .00367\,t)} \times \frac{100V}{2}\right) - 2.1$$

Values for the temperature and pressure correction factor are given in the table on page 453. The following equation may also be used.

$$\text{Volume per cent } O_2 \text{ capacity} = \left(\frac{17.9(B - w)V}{t + 273}\right) - 2.1$$

The value 2.1 in these equations corrects for oxygen and nitrogen physically dissolved at atmospheric pressure. For oxygen content subtract 1.36 (per cent N_2) instead of 2.1. For O_2 combined with hemoglobin subtract 1.7 or 1.5 for arterial or venous blood respectively, which corrects for free O_2 and N_2 at arterial or venous tension. O_2 unsaturation is obtained by subtracting O_2 combined with hemoglobin from O_2 capacity.

Hemoglobin (gm. per 100 c.c.) = 0.746 × volume per cent O_2 capacity.

11. Colorimetric Determination of Hemoglobin.—In spite of the great clinical importance of hemoglobin determinations, they are as a rule the most poorly conducted of all blood chemical analyses. The majority of so-called office instruments in the hands of the average worker are known to give errors as high as 20 per cent. The very fact that a method is adapted for small amounts of blood should presuppose accurate calibration and use of pipettes, careful dilution and standards of definite and unchanging value.

The subject of permanent standards has occupied the attention of investigators for some time. The standard used in the acid hematin method by Cohen and Smith[1] and by Robscheit[2] is probably the best of the liquid standards. The standard used by Palmer in the carbon monoxide method[3] for hemoglobin has been shown by Robscheit to be less permanent. Newcomer[4] has recommended a brown glass plate of definite thickness which has been standardized spectrophotometrically to match the acid hematin color. These plates may be fitted into a Duboscq type of colorimeter. The Newcomer hemoglobinometer[5] is a Duboscq type of microcolorimeter provided with such a glass plate.

[1] Cohen and Smith, *Jour. Biol. Chem.*, 38, 489, 1919.
[2] Robscheit, *Jour. Biol. Chem.*, 41, 209, 1920.
[3] Palmer, *Jour. Biol. Chem.*, 33, 119, 1918.
[4] Newcomer: *Jour. Biol. Chem.*, 55, 569, 1923.
[5] Manufactured by the Bausch and Lomb Optical Co., Rochester, N. Y. This firm also supplies the brown glass plates.

All acid hematin methods depend on the use of standards which are prepared on the basis of oxygen capacity determinations of blood. These determinations should be conducted frequently to check possible variations in the "permanent" standards. It is also good practice to check the manufacturer's standardization of glass plates by this method, but this should not be attempted by one unfamiliar with the technique of gasometric analysis.

Wong[1] has devised a hemoglobin method in which the iron in the pigment is estimated colorimetrically after oxidation. This appears to be quite an exact procedure and is a departure which warrants more general trial.

It has been the custom among physicians to express hemoglobin values in terms of per cent of some arbitrarily established normal. Numerous workers, using as many methods, have tried to determine what is normal and the result has been that at least a half dozen different concentrations of hemoglobin are accepted as normal (100 per cent) by practitioners. The logical way to avoid this chaotic state, it would seem, is to express hemoglobin values in terms of actual concentration, without committing oneself to any particular scale of normals.[2] Thus 14.5 per cent (*i.e.*, gm. per 100 c.c. of blood) hemoglobin means something very definite, while the same concentration is 105 per cent on one arbitrary scale (Haldane) and 86 per cent on another (Williamson).

Normal hemoglobin concentrations as determined by various investigators, and the methods used are:

13.8 per cent	Haldane[3]	Oxygen capacity
16.9 per cent	Williamson[4]	Spectrophotometer
15.6 per cent	Haden[5]	Oxygen capacity, recalculated on the basis of a normal red cell count of 5,000,000.

Of these, the last is probably the most acceptable figure and is recommended for general adoption. Tables giving the concentration of hemoglobin in 100 c.c. of blood corresponding to colorimetric readings are very convenient, and simple to construct.

Acid Hematin Method. Principle.—The depth of color of acid hematin resulting from the dilution of the blood in hydrochloric acid, is compared with (a) a standard acid hematin solution, or (b) a standardized brown glass plate.

Procedure.—0.05 c.c. of blood from a freely flowing source are measured by means of a calibrated micropipette into 10 c.c. of approximately 0.1 N hydrochloric acid. The blood is rinsed out thoroughly by sucking up the acid and

[1] Wong: *Jour. Biol. Chem.*, 55, 421, 1923.

[2] A plea for such standardization has been made before the Am. Soc. of Clinical Pathologists by Rice (*Jour. Lab. Clin. Med.*, 11, 737, 1926).

[3] Haldane: *Jour. Physiol.*, 26, 497, 1900–01.

[4] Williamson: *Arch. Int. Med.*, 18, 505, 1916.

[5] Haden: *Jour. Amer. Med. Assn.*, 74, 1496, 1922.

blowing out several times. If the blood appears to be low in hemoglobin, it is advisable to use a double sample. Mix well and let stand for at least an hour. If an earlier report is essential, place the tube in a warm water bath for ten minutes. The solution is then read in the colorimeter, using either of the standards described below. (a) Standard acid hematin solution.[1] A large sample of blood is collected in an oxalated tube and its oxygen capacity determined by either of the procedures of Van Slyke (pp. 452 and 466). On the basis of the Haldane figure (1 volume per cent O_2 = 0.746 gm. hemoglobin) calculate the hemoglobin concentration, and dilute the blood with 0.1 N HCl in a volumetric flask so that the resultant hemoglobin (acid hematin) concentration is 3 per cent. That is, if the blood has a hemoglobin concentration of 15 per cent, dilute 20 c.c. to 100 c.c. with 0.1 N HCl. If the hemoglobin concentration is 14.2 per cent, dilute 21.1 c.c. $\left(\frac{20 \times 15}{14.2}\right)$ to 100 c.c. with the acid. The 3 per cent solution thus obtained is well mixed and kept in a refrigerator. It will not deteriorate for at least three months. From this stock solution the comparison standard is prepared fresh every week, by diluting 5 c.c. to 200 with 0.1 N HCl, making an acid hematin solution equivalent to 0.075 per cent hemoglobin. (b) Standard Newcomer Plate.[2] The factor accompanying the glass plate as obtained from a manufacturer may be checked against an acid hematin solution from a specimen of blood whose hemoglobin concentration has been determined by oxygen capacity. The comparison is made by placing the plate either in the slit above the plunger at the left, or in the support below the left cup, in either case immersing the plunger to a depth of about 15 mm. into water contained in the cup. The acid hematin solution is then read against the standard glass plate. In the Newcomer hemoglobinometer the plate is contained within the prism box.

Calculation.—(a) Standard acid hematin solution:

$$\text{Grams Hemoglobin per 100 c.c.} = \frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.075 \times \frac{100}{0.05} \times \frac{10}{100}$$

If the unknown is set at 15 on the left, the reading of the standard gives directly the per cent hemoglobin. If other dilutions of blood or concentrations of standard are employed the above equation must be corrected accordingly, and tables may be constructed giving the hemoglobin concentration corresponding to each colorimeter reading for the conditions used.

(b) Standard Newcomer Plate: For a plate exactly one mm. thick, Newcomer[2] gives the following formula:

$$\text{Per cent Hemoglobin (gm. per 100 c.c.)} = \frac{0.38}{\text{Reading of Unknown}} \times \text{dilution.}$$

The dilution in the above method is 200 $\left(\text{i.e., } \frac{10}{0.05}\right)$. The factor 0.38 (plate factor) must be corrected if the thickness varies from 1 mm., by referring to correction tables of Newcomer in the article cited (1919). If the factor has been determined for a particular plate by the manufacturer, it should be substituted for the value given in the formula. The best procedure, however, is to calibrate one's own plate by comparison with a standard acid hematin solution. In this case a constant may be obtained by multiplying the plate factor obtained by

[1] Cohen and Smith: *Jour. Biol. Chem.*, 39, 489, 1919; modified by Bernard L. Oser.
[2] Newcomer: *Jour. Biol. Chem.*, 37, 465, 1919; 55, 569, 1923.

the dilution, and into the product the colorimetric reading of the unknown is divided to obtain the hemoglobin concentration. A convenient table may be made giving the colorimetric readings for the hemoglobin range usually encountered.

12. **Determination of Carbon Monoxide in Blood.**[1] **Principle.**—A more exact quantitative procedure for the estimation of carbon monoxide than the colorimetric method given on page 416, is a gasometric method based on the experiments of Van Slyke and his associates.[2] The mixed gases are extracted from the sample of blood in a Van Slyke pipette (Fig. 130) the oxygen and carbon dioxide are absorbed, and the carbon monoxide estimated in the residual gas either by absorption with Winkler's reagent or by correction for the nitrogen.

Procedure.—Blood should be collected under oil in an oxalated tube. A special CO reagent[3] is required which differs from the O_2 reagent in containing lactic acid as well as more potassium ferricyanide. The reagent (7.5 c.c.) is made gas-free and the mixed gases are extracted from 2 c.c. of blood exactly as described under the determination of oxygen capacity (p. 452). Longer shaking may be required to arrive at constant volume, since CO is not so readily dissociated from its combination with hemoglobin. After the extraction, a negative pressure is created in the extraction chamber by raising the solution level to a point 2 to 3 cm. below the 2.5 c.c. graduation. About 0.5 c.c. of mineral oil are introduced into the cup and below this 1 c.c. of alkaline pyrogallate reagent.[4] The reagent is admitted until its level reaches the capillary of the cup, and the column that forms in the graduated capillary is broken by means of a stream of mercury as described in the determination of oxygen capacity. A few minutes are allowed for drainage, the gas restored to atmospheric pressure and the volume read. The absorption is repeated until the latter is constant. The CO may be estimated from this volume reading, the temperature and barometric pressure.

If it is desired to determine the CO directly by absorption, proceed from this point by first drawing the mixed solutions in the pipette into reservoir *d*. This is to avoid mixing the alkaline pyrogallate solution with Winkler's reagent[5] to be used. Establish a slight negative pressure within the extraction chamber as described above. Rinse the delivery cup with distilled water and introduce into the pipette 0.5 c.c. Winkler's reagent. Absorption of CO takes place rapidly and the final gas volume should be read at once.

[1] For tannic acid method see p. 416. A modified tannic-pyrogallic acid method is described by Sayers and Yant. Bureau of Mines, Technical Paper 373, 1925.

[2] Adapted by Bernard L. Oser.

[3] *Special CO Reagent:*

Saponin	3.0 gm.
Potassium ferricyanide	8.0 gm.
Lactic acid c.p.	4.0 c.c.
Caprylic alcohol	3.0 c.c.
Distilled water to	1000 c.c.

[4] *Alkaline Pyrogallate Reagent:*

Prepare a solution of potassium hydroxide by dissolving 160 gm. in 130 c.c. water. In 200 c.c. of this solution dissolve 10.0 gm. pyrogallic acid.

[5] *Winkler's Reagent:*

Cuprous chloride	40 gm.
Ammonium chloride	50 gm.
Distilled water to	150 c.c.

For use mix this solution with ammonium hydroxide (sp. gr. 0.9) in the proportion of 3:1.

A method of magnifying small volumes of gas by reducing the pressure a definite amount below atmospheric, is described by Van Slyke and Stadie.[1]

Larger samples of blood may be used, the amounts of reagents being proportionately increased.

Calculations:

V_1 = observed volume of gas from 2 c.c. of blood, after absorption by alkaline pyrogallate solution.

V_2 = Same, after absorption by Winkler's reagent.

B = Barometric pressure in mm. Hg.

w = Tension of aqueous vapor in mm. Hg. (See table in Appendix.)

t = temperature in °C.

$$\text{Volume per cent CO} = \left(\frac{(B-w)}{760(1+.00367t)} \times \frac{100V_1}{2}\right) - 1.36$$

$$\text{or Volume per cent CO} = \left(\frac{(B-w)}{760(1+.00367t)}\right)\frac{100(V_1-V_2)}{2}$$

Values for the temperature and pressure correction factor may be found in the table on page 453.

MANOMETRIC METHODS OF ANALYSIS OF GASES IN BLOOD AND OTHER SOLUTIONS[2]

Principle.—The manometric methods of gas analysis differ from the more commonly used volumetric methods in that the latter involve reading the volume with the gas under a definite (usually atmospheric) pressure, whereas by manometric methods the pressure required to keep the gas at a fixed volume is observed. The advantages of this procedure are that a much lower degree of error is attainable, since in the former method the error in volume reading is many times greater than the error in reading of barometric pressure; calculation is greatly simplified, as barometric pressure and corrections for vapor tension and capillary attraction of mercury do not enter in; smaller quantities of material may be used; and accuracy is attainable over a wide range of gas concentration.

Apparatus.—The apparatus consists of a short pipette with the upper stem closed by a stop-cock, the lower connected with a glass tube. The latter descends 800 mm., then turns at a right angle to connect with a levelling bulb and a mercury manometer, which may be either open at the upper end (Figs. 135 and 136) or closed (Fig. 137). The pipette is calibrated at two points to hold a c.c. of gas for pressure measurement and A c.c. of total volume, respectively, as shown in Fig. 136.

For analysis the sample of blood or other solution is introduced into the chamber over mercury, together with the reagents to free the desired gases from combination. A Torricellian vacuum is obtained, as in the "volumetric" apparatus, by lowering the levelling bulb, and the gases are extracted from solution by 2 or 3 minutes shaking. The gas volume is then reduced to a c.c. by admission of mercury through cock c, and the reading p_1 is made on the manometer. The gases are either ejected or are absorbed by proper reagents, and the reading p_2

[1] Van Slyke and Stadie: *Jour. Biol. Chem.*, 49, 1, 1921.

[2] Van Slyke, and Neill: *J. Biol. Chem.*, 61, 523, 1924. In addition to the methods here to be described, this paper gives the details of the technique for the combined determination of all the gases in a sample of blood; methods for micro analyses of fractions of a c.c. of blood; for determination of gases in liquids saturated at high tensions; for determination of dissolved gases in water; and for the use of the apparatus in air and general gas analyses. Harington and Van Slyke: *Ibid.*, 61, 575, 1924, have modified the form of the extraction chamber to permit the removal of successively added reagents before each gas measurement. A technique is described which eliminates reabsorption corrections for CO_2 and CO.

is taken, with the same gas volume. The partial pressure P of the gas at a c.c. volume is then $P = p_1 - p_2$ mm. of mercury, from which the gas volume at 0°, 760 mm. may be calculated.

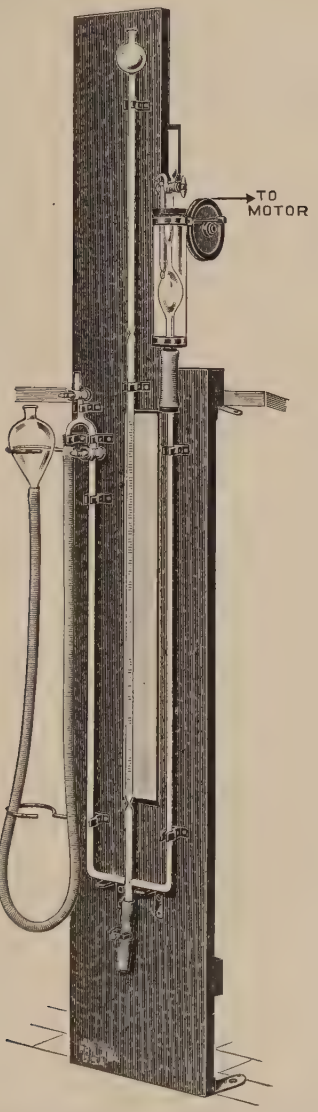


FIG. 135.—VAN SLYKE AND NEILL MANOMETRIC GAS ANALYSIS APPARATUS. (OPEN TYPE.)

The extraction chamber differs from that of the "volumetric" apparatus in being calibrated at only three points, viz., 0.5, 2.0 and 50 c.c., and in having no cock at the bottom. The greater accuracy of readings by this method necessitates the use of a mercury seal around the rubber joint at the bottom of the extraction chamber (See Fig. 138.) A mechanical shaker (Stadie, *Jour. Biol. Chem.*, 49, 43, 1921) is provided. Air which diffuses through the rubber tubing

of the levelling bulb is expelled through stop-cock *d*. The screw-clamp at the bottom of the manometer permits withdrawal of the mercury.

While there is a slight theoretical advantage in favor of the closed type of manometer, the open type is simpler to operate and will probably fall into more general use. The following methods will, therefore, be described with the latter in mind.

For calibration of the apparatus and a method of constructing a simple but not necessarily inaccurate modification of the manometric apparatus, see the original paper.

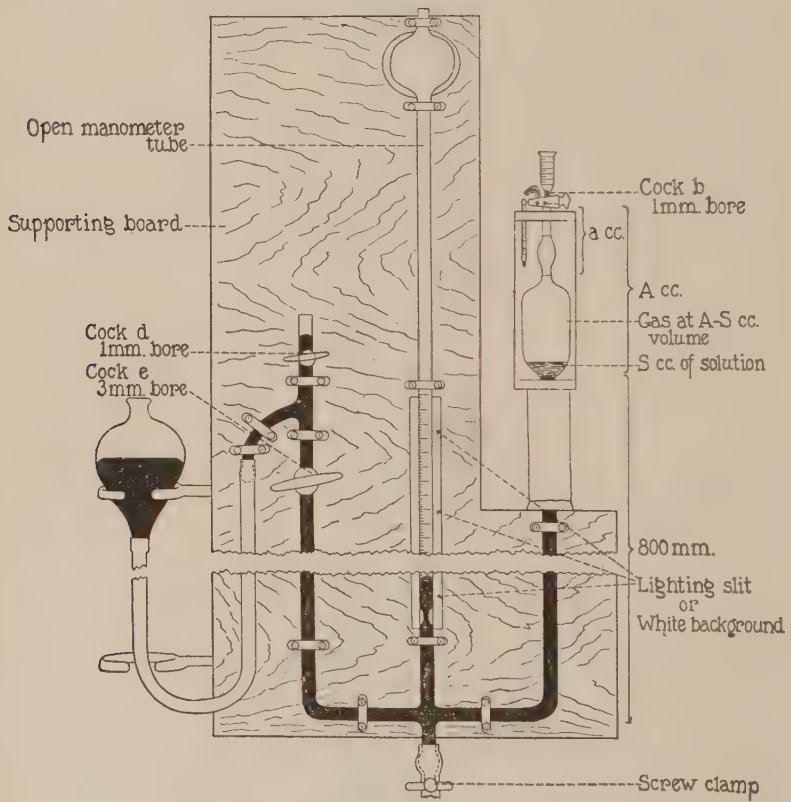


FIG. 136.—OPEN TYPE OF MANOMETRIC APPARATUS.

Measuring Samples.—In order to attain the advantage of precision offered by the manometric apparatus accuracy in collecting and measuring samples is essential. Blood should either be drawn directly from the vein, without stasis into the differential pipette from which it is to be delivered into the extraction chamber, or better collected under oil in an oxalated tube from which it can be drawn as needed. The corpuscles should be uniformly distributed with a fine stirring rod before each sample is withdrawn. A heavy walled Ostwald pipette graduated to deliver between two marks ("differential" pipette) and preferably provided with a stop-cock should be used (See Fig. 139).

Blood must be admitted at a rate slow enough to permit clear drainage of the pipette. Smooth delivery is usually obtained by using cock *e*, the mercury

surface in the open manometer at the start being adjusted at about 2 cm. above that in the chamber. An ordinary transfer pipette may be used, the final drop of blood being expelled by keeping the tip immersed, closing the mouth of the pipette with the index finger and grasping the bulb with the warm palm of the other hand. The expanding air drives out the remaining blood.

FIG. 137.—CLOSED TYPE OF MANOMETER TUBE.

Cleaning the Apparatus.—Preceding each analysis, the apparatus is cleaned by introducing 10 or 15 c.c. of approximately 0.01 N lactic acid, shaking for 15 or 20 seconds in the evacuated chamber and ejecting the extracted gas and solution. Adherent solution introduces no error in the subsequent analysis.

Occasionally the apparatus should be kept filled over night with chromic-sulphuric acid mixture, admitted through cock *b* by regulation of cock *e*. The latter is kept closed but the former open to allow the escape of gas.

Testing for Leaks.—Test for leaks by introducing S c.c. of water (Fig. 136) and extracting dissolved air for 2 minutes. Reduce gas volume to a and read pressure. If temperature is constant the pressure should not increase when this extraction is repeated.

Lubrication.—Cocks b and e must turn smoothly but not leak. A thin layer of vaseline is first applied, followed by a rubber paste (made by dissolving 1 part unvulcanized rubber in 5 parts vaseline, with heat), using relatively less of the first coating in warm weather.

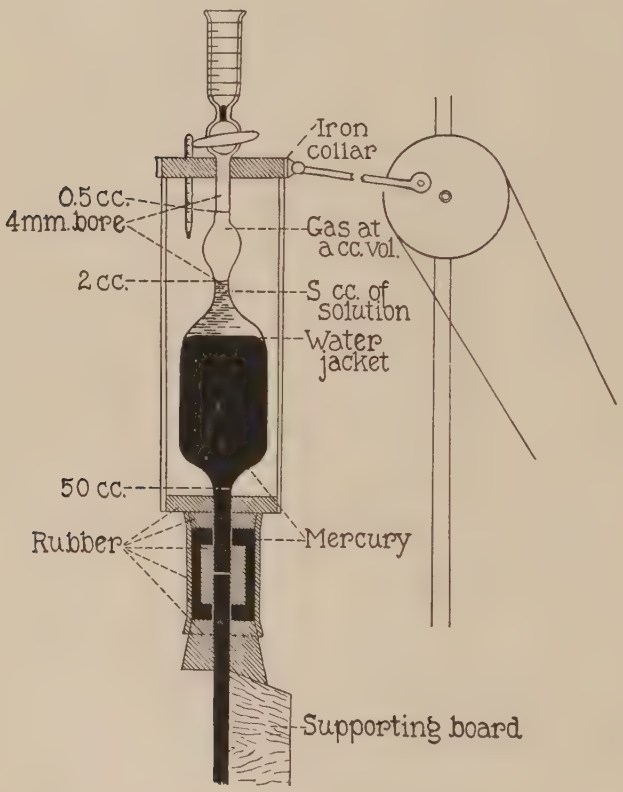


FIG. 138.—EXTRACTION CHAMBER.

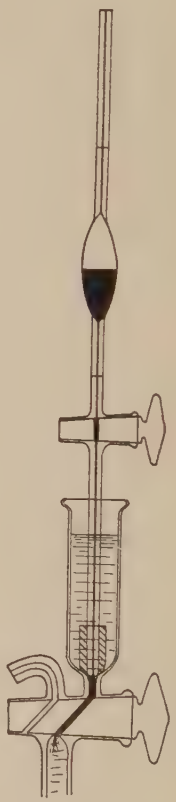


FIG. 139.—OPERATION OF DELIVERY PIPETTE.

Determination of Correction, c, for Manometer Depression Caused by Introduction of Absorbent Solution.—The introduction of absorbent solution causes a lowering of the mercury meniscus in the chamber and hence in the manometer, by increasing the volume of fluid between the mercury and the a mark at the moment of reading (Fig. 136). This necessitates for the p_2 reading a correction, which is determined by blank analyses. The shape of the apparatus causes the area of the meniscus of the mercury in the chamber to vary according to the values of S and a. The value of c for 1 c.c. of added solution may accordingly be from 1 to 4 mm., depending on the shape of the chamber and the volume of S.

Dilute absorbents (N NaOH) or hydrosulphite (20 per cent solution) have no significant effect on the vapor pressure in the chamber. Special precautions are required for alkaline pyrogallate solution (p. 457) because of the strong KOH.

When the final manometer reading is obtained after expulsion of the gases instead of after addition of an absorbing solution, c, of course, is zero.

Calculation.—The general equation for calculating total gas content of a solution from the volume of gas extracted in an evacuated chamber of definite volume, as developed by Van Slyke and Stadie[1] has been subjected to certain modifications and made adaptable to manometric calculations.

Vapor pressure is eliminated as a factor since it is the same for both pressure readings and cancels out in the equation $P = p_1 - p_2$. An i correction for reabsorption of extracted gas during the release of the vacuum, is required for CO_2 just as in volumetric measurements, and amounts to about 1.014. For less soluble gases (O_2, N_2, CO) it is practically 1.000, reabsorption being negligible. A correction is also included for the effect of temperature on the specific gravity of mercury.

FACTORS FOR CALCULATION OF VOLUMES PER CENT OF BLOOD GASES FROM PRESSURES IN 50 c.c. APPARATUS[2]

Temperature	Factors for CO_2			Factors for O_2, CO, and N_2				
	Sample = 0.2 c.c. S = 2.0 c.c. a = 0.5 c.c. i = 1.03	Sample = 1 c.c. S = 3.5 c.c. a = 2.0 c.c. i = 1.014	Sample = 2 c.c. S = 7.0 c.c. a = 2.0 c.c. i = 1.014	Sample = 0.2 c.c. S = 2.0 c.c. a = 0.5 c.c. i = 1.00	Sample = 1 c.c. S = 3.5 c.c.		Sample = 2 c.c. S = 7 c.c.	
					a = 0.5 c.c. i = 1.00	a = 2.0 c.c. i = 1.00	a = 0.5 c.c. i = 1.00	a = 2.0 c.c. i = 1.00
°C.								
15	0.335	0.2725	0.1483	0.312	0.0623	0.2493	0.0317	0.1251
16	33	11	70	10	21	85	15	46
17	31	0.2697	59	09	19	78	14	42
18	30	83	49	08	17	68	12	37
19	28	69	39	07	15	59	11	32
20	27	55	29	07	13	50	09	28
21	26	40	19	06	10	41	08	24
22	24	26	10	05	08	32	06	19
23	23	13	01	03	06	23	05	15
24	22	00	0.1391	02	04	14	03	10
25	20	0.2588	82	01	02	06	02	06
26	18	75	73	00	00	0.2398	01	02
27	17	62	64	0.299	0.0598	90	0.0299	0.1198
28	16	49	56	98	96	82	98	93
29	14	37	49	97	93	74	96	89
30	13	26	41	96	92	66	95	85
31	12	15	33	95	90	58	94	81
32	11	04	25	94	88	50	92	77
33	10	0.2493	18	93	86	42	91	73
34	08	82	10	92	83	33	90	69

[1] *Jour. Biol. Chem.* 49, pp. 30 and 31, 1921.

[2] If calibration of an apparatus shows a value of a significantly different from the 0.500 or 2.000 c.c. in the column heading, the factors in the column are corrected by multiplying them by $\frac{a}{0.500}$ or $\frac{a}{2.000}$.

The use of the rather involved final equation is expedited by the tables constructed by the authors, which give directly the factors by which P, read at any given temperature under the conditions ordinarily employed, must be multiplied to obtain the volumes per cent of gas (see table p. 463). To express results as millimols per liter, either use the table of millimol per liter factors given in the original paper, or divide volumes per cent of gas by 2.24.

$$\text{Volumes per cent gas} = P \times \text{vol. per cent factor}$$

$$\text{mM gas per liter} = \frac{P \times \text{vol. per cent factor}}{2.24}$$

Determination of CO_2 in Blood or Plasma.—The apparatus having been cleaned (see p. 461), a drop of caprylic alcohol is drawn into the capillary above cock b, and 2.3 c.c. of CO_2-free water per c.c. blood or plasma to be added are put into the cup. Stop-cock b is closed, with e open and the levelling bulb at the position shown in Fig. 136. The blood or plasma is delivered beneath the layer of water in the cup from a pipette as described under "Measuring Samples." After the delivery of the sample the residue of blood in the cup is run into the chamber below, followed by the water layer. Finally 0.2 c.c. CO_2-free 0.1 N lactic acid per cc. of blood or plasma is added. Stop-cock b is then sealed

Fig. 140.—Use of Calcium Chloride Tube Reservoir.

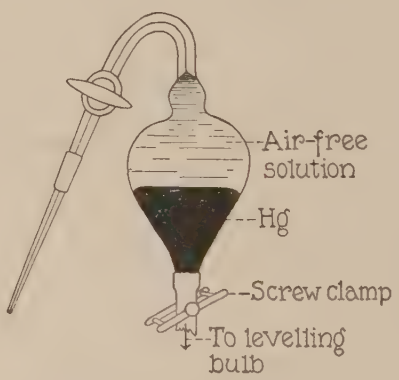


Fig. 141.—Bulb for Preparing and Storing Gas-free Solutions.

with a drop of mercury. The CO_2 is liberated by lowering the levelling bulb until the surface of the mercury has fallen to the A mark, closing cock e, and shaking the mixture for three minutes. The extracted gas is reduced to 2 c.c. (a), the admission of mercury being regulated by stop-cock e. If the fluid meniscus passes this point, readjustment must be made by first bringing the mercury meniscus to the A mark and equilibrating for a minute. Otherwise more reabsorption of CO_2 will take place than is accounted for in the calculation. The adjustment being correct, cock e is closed, the manometer tapped with the finger, and the height of the mercury column read (p_1 mm.).

The variable amounts of O_2 extracted from whole blood make it necessary to determine CO_2 by absorption. This custom is also followed in plasma analy-

ses, though it may be avoided by correcting for extracted air. The absorbent solution, 1 N NaOH[1] is admitted under reduced pressure. After measuring p_1 the pressure is diminished so that the mercury level in the chamber is somewhat below the position shown in Fig. 136, and a space of several c.c. is left between the water meniscus and the lower a mark. Cock e is closed and 2 c.c. gas-free alkali measured into the cup from the reservoir, avoiding reabsorption of air. One c.c. of alkali is admitted into the chamber. Absorption is complete in 30 seconds.

In analyses of serum or aqueous solutions of carbonates, the CO_2 may be absorbed with 0.2 c.c. of 5 N NaOH. O_2 and N_2 are sufficiently insoluble in this medium to make de-aeration unnecessary. Only slight negative pressure is required to admit it. Air-free 1 N NaOH must be used for whole blood.

After absorption of CO_2 cock b is sealed with mercury and the meniscus of the solution in the chamber is lowered a little below the a mark. The levelling bulb is placed in the position shown in Fig. 136, and mercury is readmitted from cock e until the solution meniscus is again on the a mark. The reading of the manometer is taken (p_2 mm.). The CO_2 pressure P_{CO_2} is

$$P_{CO_2} = p_1 - p_2 - c$$

where c is the correction discussed on p. 462. This correction is determined in a blank analysis in which S c.c. of water, made alkaline with 2 or 3 drops of 1 N NaOH, are extracted in the apparatus, p_1 and p_2 being read before and after addition of the same amount of alkali as used in the determination.

$$c = p_1 - p_2$$

To minimize error, all solutions must be measured very accurately, so that S varies within ± 0.05 c.c. Also sufficient time must elapse for temperature equilibrium to be established. (For other sources of error and refinements of technique see the original paper.)[2]

Calculation.—See p. 463.

Determination of Plasma CO_2 Capacity.—CO_2 capacity, as a clinical measure of acidosis, is perhaps best determined by the volumetric method of Van Slyke and Cullen (p. 432). For the manometric determination, admit a drop of caprylic alcohol into the capillary of the cup, followed by 1.5 c.c. of 0.1 N lactic acid. One c.c. of plasma (previously saturated with alveolar air) is introduced below the acid. The solutions are admitted to the extraction chamber, cock b sealed, the chamber evacuated and shaken 2 minutes. p_1 is read as described above with the gas volume at 2 c.c. The gas is ejected, the ejected portion of the solution returned to the chamber, and the pressure reduced so that the gas space is again 2 c.c. p_2 and the temperature are then read.

Calculation:

Pressure of extracted air and $CO_2 = p_1 - p_2$.

[1] The alkali must be made gas-free and kept in properly protected reservoirs. 25–30 c.c. of 1 N NaOH are de-aerated in the extraction chamber and transferred under oil to a reservoir made from a calcium chloride tube (Fig. 140). This will keep for a day. For larger amounts of solution, the apparatus shown in Fig. 141 is suggested. It consists of two leveling bulbs connected by a rubber tube 1 meter long. One of the bulbs (illustrated) is provided with a glass capillary and stop-cock. The bulb is half-filled with absorbent solution and extracted by evacuation and shaking. Solution is delivered into the extraction chamber directly from the capillary of this reservoir.

[2] Austin: *J. Biol. Chem.*, 61, 345, 1924, has described a modification of this procedure to be used in CO_2 analyses of serum obtained after ether anesthesia.

The CO_2 capacity is obtained from this pressure by the use of the nomogram (Fig. 142).

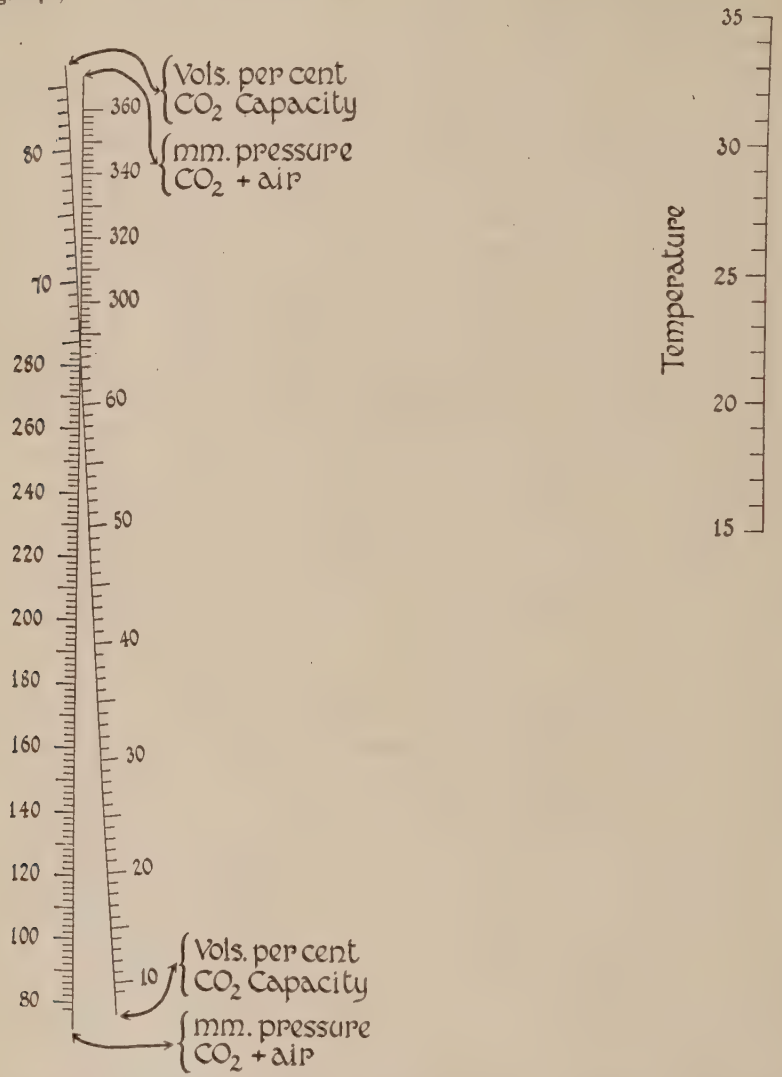


FIG. 142.—NOMOGRAM FOR CALCULATION OF PLASMA CO_2 CAPACITY. A STRAIGHT LINE CONNECTING THE OBSERVED POINTS ON THE PRESSURE AND TEMPERATURE SCALES CUTS THE CO_2 CAPACITY SCALE AT THE POINT INDICATING THE CAPACITY.

Determination of Oxygen in Blood.—Oxygen may be determined manometrically on 1 c.c. samples of blood with a variation within 0.2 volume per cent. The special O_2 reagent[1] is emulsified by rotating, and 7.5 c.c. are measured

[1] *Special Oxygen Reagent:*

Potassium ferricyanide	3.0 gm.
Saponin (Merck)	3.0 gm.
Caprylic alcohol	3.0 c.c.
Water to	1000 c.c.

Low results for oxygen may be accounted for by saponin of low hemolytic activity, in which case the amount may be increased.

into the apparatus and de-aerated by shaking in vacuo for 3 minutes. During the shaking, the mercury should be well in the neck below the bulb, to afford minimum exposure of surface, as mercury reacts with ferricyanide. One c.c. of the well mixed blood is drawn into a pipette, preferably such as described on p. 460. 6 c.c. of the extracted reagent are forced into the cup, leaving 1.5 c.c. in the chamber. The blood is introduced under the reagent directly into the chamber as described under "Measuring Samples." The pipette is carefully withdrawn and one c.c. of reagent is permitted to flow into the chamber, rinsing through the blood in the capillary. The cock is sealed with a drop of mercury and the excess reagent discarded. The apparatus is evacuated and shaken 3 minutes. 1 c.c. of air-free 1 N NaOH is placed in the cup and the CO_2 absorbed by admitting 0.5 c.c. of the hydroxide into the chamber under diminished pressure as described in CO_2 determination on whole blood.

The solution meniscus is brought to 2 c.c. (*a*) and p_1 (pressure of $O_2 + N_2$) is read. (For low O_2 values it is preferable to read p_1 with *a* at 0.5 c.c.) As in the volumetric method O_2 may now be determined either by making a correction for N_2, or directly by absorption. In the first case, after p_1 has been read, the gases are ejected from the chamber and p_2 is determined with the solution meniscus at the same *a* mark.

The absorption method is resorted to for greater precision or when other gases (*e.g.* CO) are present. After p_1 has been read, cock *e* is opened, with the levelling bulb in the position shown in Fig. 136, bringing the gas under slight negative pressure. One c.c. of hydrosulphite solution[1] is introduced into the cup. By turning cock *b*, 0.5 c.c. of the absorbent are admitted a drop at a time at 5 second intervals, consuming 2–3 minutes for the entire absorption. The solubility of N_2 in this medium is negligible. The gas is brought to the same *a* volume and p_2 is read on the manometer.

$$P_{O_2} = p_1 - p_2 - c_{O_2}$$

To check the completeness of O_2 absorption and the absence of air leakage, expel the N_2 and measure p_3 in the gas-free apparatus. The N_2 should approximate 1.2 volumes per cent in the O_2 content determination, or 1.4 volumes per cent in O_2 capacity.

An alternative absorbent for O_2 is the alkaline pyrogallate reagent (p. 457). This is not as clean or rapid as the hydrosulphite, but is more stable and its use is advised when O_2 determinations are done only occasionally. The gummy precipitate formed with the blood mixture necessitates mixing the solutions in the bulb of the chamber, well below the *a* mark, resulting in a greater negative pressure for the admission of the absorbent. One c.c. of alkaline pyrogallate is admitted dropwise over 4 or 5 minutes. The pressure is then allowed to rise to atmospheric so that the upper tube is rinsed by the solution, thus avoiding a reduction of vapor tension due to the strong alkali. p_2 is read. The apparatus is cleaned with water after using this absorbent.

The *c* correction, when oxygen is determined by absorption is due to two factors, the lowering of the mercury meniscus by the absorbent and the trace of O_2 left in the reagent after one extraction. To determine *c*, de-aerate 7.5 c.c. of the special O_2 reagent for 3 minutes, expel 5 c.c,. leaving 2.5 in the chamber.

[1] 10 gm. of powdered $Na_2S_2O_4$ are placed in a beaker, to which are added 50 c.c. of 0.5 N KOH solution. After immediate stirring, the insoluble impurity is quickly filtered off through cotton. The solution is at once de-aerated and transferred to a container under oil as described in the footnote on p. 465.

Shake again for 3 minutes and then admit 1.5 c.c. of air-free 1 N NaOH in the manner described for CO_2. Read p_1 with a at 0.5 and 2.0 c.c. Then run in the absorbent as in the analysis and obtain p_2 at both points. Use c_{CO_2} corresponding to a in the analysis.

$$c_{O_2} = p_1 - p_2$$

Eject residual N_2 and read p_3

$$c_{N_2} = p_2 - p_3$$

If O_2 is determined indirectly by correcting for N_2 instead of by absorption, c is obtained in the same way, except that p_2 is measured after ejection of the $O_2 + N_2$, without preliminary absorption.

Calculations:

The estimated corrections for dissolved O_2 and N_2 of blood in calculation of total or combined oxygen or oxygen capacity, given in the following table may vary somewhat with the cell content.

ESTIMATED CORRECTIONS FOR DISSOLVED O_2 AND N_2 IN BLOOD

Blood	Determined	Sought	Correction to subtract	
			Vol. per cent	mM. per l.
Venous	Total O_2	Combined O_2	0.1 (O_2)	0.04 (O_2)
Arterial	Total O_2	Combined O_2	0.2 (O_2)	0.09 (O_2)
Saturated with air at 20°, 760 mm	Total O_2	Combined O_2 (O_2 capacity)	0.5 (O_2)	0.22 (O_2)
Venous	Total $O_2 + N_2$	Combined O_2	1.3 ($O_2 + N_2$)	0.57 ($O_2 + N_2$)
Arterial	Total $O_2 + N_2$	Combined O_2	1.5 ($O_2 + N_2$)	0.62 ($O_2 + N_2$)
Saturated with air at 20°, 760 mm.	Total $O_2 + N_2$	Combined O_2 (O_2 capacity)	1.9 ($O_2 + N_2$)	0.85 ($O_2 + N_2$)
Venous	Total $O_2 + N_2$ or $CO + O_2 + N_2$	Total O_2 or $CO + O_2$	1.2 (N_2)	0.53 (N_2)
Arterial	Total $O_2 + N_2$ or $CO + O_2 + N_2$	Total O_2 or $CO + O_2$	1.2 (N_2)	0.53 (N_2)

The corrections are subtracted from the volume per cent of gas as determined by the use of the table on p. 463.

Determination of Carbon Monoxide in Blood.—Carbon monoxide is liberated from combination with hemoglobin by treatment with an acidified ferricyanide reagent (p. 457). Of the extracted gases, the CO_2 and O_2 are absorbed with alkaline hydrosulphite and the CO determined by correcting for N_2 in the residual gas. 10 c.c. of the reagent are de-aerated and admitted to the cup. Two c.c of blood are transferred directly from the pipette to the extraction chamber and extracted in the evacuated chamber for three minutes with 5 c.c. of the special CO reagent, the remaining 5 c.c. being discarded. The CO_2 and O_2 are absorbed by 1 c.c. of an alkaline hydrosulphite solution[1] added in small portions during 3 or 4 minutes under slight negative pressure. The methemoglobin formed by the action of ferricyanide is rapidly reduced by hydrosulphite to reduced hemoglobin, which combines readily with CO or O_2. This reabsorption can be kept at a constant minimum, however, by avoiding unnecessary agitation, and approximates 2.4 per cent of the total CO present. This factor should be determined by

[1] For this purpose the hydrosulphite is made up in 1.0 N KOH instead of the 0.5 N KOH ordinarily used (p. 467).

the analysis of blood saturated with illuminating gas. The hydrosulphite is run into the chamber without agitation of the solution, the levelling bulb resting in the position shown in Fig. 136, cock *e* being open. As soon as O_2 absorption is complete, cock *e* is closed and the levelling bulb placed in the lowest position. Cock *e* is then gradually opened, lowering the level of the fluid to either the 0.5 or 2.0 c.c. mark, depending on whether little or much CO is present. The same precautions against overrunning the mark must be taken here as in the CO_2 determination. The liquid level should be lowered slowly enough to allow complete drainage. The pressure p_1, representing CO + N_2, is read. The gas is then ejected and pressure p_2 read.

Calculation: The CO + N_2 pressure is calculated as

$$P = 1.024\ (p_1 - p_2)$$

in which 1.024 represents the reabsorption factor, which should be estimated by each analyst under the conditions of the determination. From P, the CO + N_2 content is calculated in the table on p. 463 and 1.2 volumes per cent are subtracted for the N_2.

Volumes per cent CO = $P \times$ (vol. per cent factor) − 1.2.

Determination of Methemoglobin in Blood.[1]—While CO alone changes only active (reduced and oxy-) hemoglobin to carboxyhemoglobin, the simultaneous action of hydrosulphite and CO causes the same change to take place with methemoglobin also. Therefore it is possible to estimate the methemoglobin from the difference in CO-combining power before and after treatment of the blood with hydrosulphite. Place in a 100 c.c. cylindrical separatory funnel exactly 5 or 10 c.c. of blood and one-fifth as great a volume, accurately measured, of freshly prepared ammoniacal hydrosulphite solution,[2] making blood solution A. The air in the funnel is immediately replaced by flushing thoroughly with CO at atmospheric pressure.[3] In a second similar funnel are placed the same volume of blood, one-fifth the volume of water and an atmosphere of CO, to make blood solution B. Both vessels are now rotated for 15 minutes. Samples for analysis should be drawn immediately after opening the funnels to the air. Carbon monoxide determinations are conducted as described above, substituting air-free 1 N NaOH as the absorbent instead of hydrosulphite, since all the O_2 has been replaced by CO. After the CO_2 is absorbed, p_1 is measured. The gas is then ejected from the chamber and p_2 is read.

Calculation: In each case the CO pressure is

$$P_{CO} = p_1 - p_2.$$

The carbon monoxide contents are calculated as described above, correcting for dilution with hydrosulphite or water by multiplying each result by 6/5. The methemoglobin, in terms of CO or O_2 capacity, is calculated from the equation

$$\text{Methemoglobin} = (\text{CO in A}) - (\text{CO in B}).$$

[1] Van Slyke: *Jour. Biol. Chem.*, 66, 409, 1925.

[2] Prepared according to Nicloux (*Bull. Soc. chim. biol.*, 6, 728, 1924). Pour 50 c.c. of diluted (1:50) ammonia solution upon 2.0 gm. of pulverized $Na_2S_2O_4$ contained in a 100 c.c. beaker. Cover at once with a 1 cm. layer of paraffin oil. Dissolve the hydrosulphite by stirring with a rod for a few seconds.

[3] CO may be prepared by dropping concentrated sulphuric acid upon crystals of oxalic acid or into anhydrous formic acid and warming with a burner, the evolved gas being collected in a gasometer. If oxalic acid is used the gases must be washed with alkali to remove CO_2. 1 gm. oxalic acid yields 275 c.c. CO. 1 c.c. formic acid yields 500 c.c. CO. For methemoglobin determinations it is possible to use illuminating gas instead of pure CO.

The total hemoglobin content may be determined from the equation

Total Hemoglobin = (total CO in A) − (physically dissolved CO).

For an indirect estimation of the latter correction see the original paper. It is advised to use pure CO and not illuminating gas if the total hemoglobin is desired.

Determination of Urea in Blood.[1]—By the action of urease, urea is converted to ammonium carbonate which is commonly estimated by nesslerization or titration of the ammonia. It is possible, however, to estimate the CO_2 which can be liberated from the ammonium carbonate upon treatment with an acid, and thus get a measure of the urea. The entire determination may be performed in the manometric apparatus, as the blood proteins protect the urease from inactivation by mercury. One c.c. of blood and 1 c.c. of 0.1 N lactic acid[2] are introduced into the extraction chamber, which is evacuated and shaken 1 minute to remove preformed CO_2. 0.5 c.c. of 20 per cent urease solution[3] and 0.5 c.c. of 0.3 M Na_2HPO_4 are then added.[4] The mercury in the chamber is lowered and raised again, to bring the urease into contact with the blood mixture wetting the walls. After standing until digestion is complete (5 minutes or more), 0.5 c.c. of 1 N lactic acid is added, and the determination continued as described in the method for CO_2.

Calculation: A concentration of 1 millimol of CO_2 per liter of blood is equivalent to 1 millimol or 60 mg. of urea, or 28 mg. of urea N per liter. Or, since 1 c.c. of CO_2 is equivalent to $\frac{28}{22.4}$ mg. N,

mg. urea N per 100 c.c. blood = (Volumes per cent Urea-CO_2)1.25.

Determination of Urea in Urine.[5]—The same principle is employed for this determination as for urea in blood, except that preformed CO_2 is removed by acidification with phosphoric acid, which is later neutralized with sufficient alkali to form a proper buffer medium for the urease action. Into a 20 c.c. rubber-stoppered flask are placed 2 c.c. of urine of specific gravity below 1.030 or 1 c.c. of more concentrated urine. 0.25 c.c. of 2 M H_3PO_4[6] is added, and the mixture whirled about the walls of the flask to permit the escape of CO_2, following which it is diluted to 18 c.c. 0.35 c.c. of CO_2-free 2 M NaOH[7] are added from a capillary tip with which the reservoir is fitted. Then 1 c.c. of urease solution[8] is

[1] Van Slyke: *Proc. Soc. Exp. Biol. and Med.*, 22, 486, 1925.

[2] Made by diluting 1 volume of commerical lactic acid (specific gravity 1.20) to 100.

[3] Prepared from jack bean meal by the acetone precipitation method of Van Slyke and Cullen (*J. Biol. Chem.*, 19, 211, 1914) (See Urea Method (1) Chapter XXXI). This preparation is CO_2-free. Any other CO_2-free urease may be used if preliminary tests show it to have sufficient activity.

[4] Prepared by dissolving 5.34 gm. Sørensen's disodium phosphate ($Na_2HPO_4 \cdot 2H_2O$) in water and making up to 100 c.c.

[5] Van Slyke: Personal communication.

[6] Dilute 6.7 c.c. of syrupy H_3PO_4 (sp. gr. 1.725) to 50 c.c.

[7] Prepare a stock alkali solution by mixing 100 gm. of stick NaOH with an equal weight of distilled water until dissolved, and allowing to stand in a stoppered flask until the insoluble Na_2CO_3 has settled. This solution is approximately 18 N. It should be protected from atmospheric CO_2 by a soda-lime tube. To prepare the 2 M NaOH used in the determination, 5.5 c.c. of this stock alkali are diluted to 50 c.c. and drawn into a calcium chloride tube (Fig. 139) protected at the top by a soda-lime tube. The H_3PO_4 and NaOH to be used, may be tested by adding one c.c. of each solution to 20 c.c. of water, dividing this solution into two parts, to one adding methyl orange and to the other alizarin. The solution should be alkaline to the first and acid to the second indicator (pH between 4 and 5).

[8] A 10 per cent solution of Squibb's urease prepared from jack beans by the acetone precipitation method of Van Slyke and Cullen.

introduced from a pipette dipping below the surface of the diluted urine. Without agitating the heavy urease solution which sinks to the bottom, the solution is diluted to 19.9 c.c. and the flask closed with a 1-hole rubber stopper, the hole of which is plugged with a glass rod. The contents of the flask are mixed and digestion allowed to proceed for 20 minutes or more. The plug is withdrawn from the stopper and 0.2 c.c. of the CO_2-free 18 N NaOH are added through the hole in the stopper. Greasing the delivery tip of the pipette will permit the alkali to fall cleanly from the end without creeping back up the sides. The 0.2 c.c. of alkali increases the total volume to only 20 c.c. The stopper is again plugged and the flask inverted a few times. The solution is now sufficiently alkaline to prevent

FACTORS BY WHICH MILLIMETERS OF PRESSURE FROM UREA CO_2 ARE MULTIPLIED TO GIVE UREA CONTENT OF URINE

Temperature	Grams urea N per liter urine		Grams urea per liter urine	
	Sample = 0.1 c.c. urine	Sample = 0.2 c.c. urine	Sample = 0.1 c.c. urine	Sample = 0.2 c.c. urine
15	0.0341	0.01707	0.0731	0.0365
6	40	00	28	64
7	38	0.01690	24	62
8	36	82	20	60
9	34	72	16	58
20	33	64	12	56
1	31	54	08	54
2	29	45	05	52
3	27	37	01	51
4	26	30	0.0698	49
25	24	22	94	47
6	23	13	91	45
7	21	05	87	44
8	20	0.01598	84	42
9	18	91	81	41
30	16	84	78	39
1	15	77	75	38
2	14	70	72	36
3	13	63	69	35
4	11	56	66	33

escape of CO_2, but the flask should be kept closed between taking samples to prevent absorption of atmospheric CO_2. The actual estimation of CO_2 formed from the urea is performed as follows. A drop of caprylic alcohol is placed in the capillary and 1 c.c. of water in the cup of the manometric apparatus. 2 c.c. of the digested urine are run under the water layer and the contents of the cup admitted to the chamber. 0.5 c.c. of 1 N lactic acid[1] are introduced, the cup

[1] Dilute 1 volume of commercial lactic acid (sp. gr. 1.20) to 10 volumes with water.

sealed with mercury and the chamber evacuated and shaken 1.5 minutes. Reduce the gas volume to 2 c.c. (a) as described for CO_2 in blood, and read p_1 on the manometer. Eject the solutions from the apparatus and run a control with the reagents alone, but without urine. The manometer reading for the control is p_0. One control serves for a whole series of determinations. If the temperature of the test differs from the control, add or subtract 1 mm. to p_0 for each degree rise or fall, provided the difference is not greater than 3°. It is unnecessary to clean the apparatus between determinations when running a series. One may, therefore, proceed with analyses at the rate of one every 5 minutes. The addition of 5 drops of 0.04 per cent bromcresol purple at the same time with the urease, enables one to follow the stage of the digestion by the color changes.

Calculation: Pressure due to Urea $CO_2 = p_1 - p_0$

The factors for calculating urea or urea nitrogen from this pressure are given in the above table.

CHAPTER XX

THE ENDOCRINE ORGANS[1]

The secretions of the endocrine glands (see Fig. 143) pass directly into the blood stream. These secretions are necessary for the normal functioning of the body. During the last few years the knowledge of

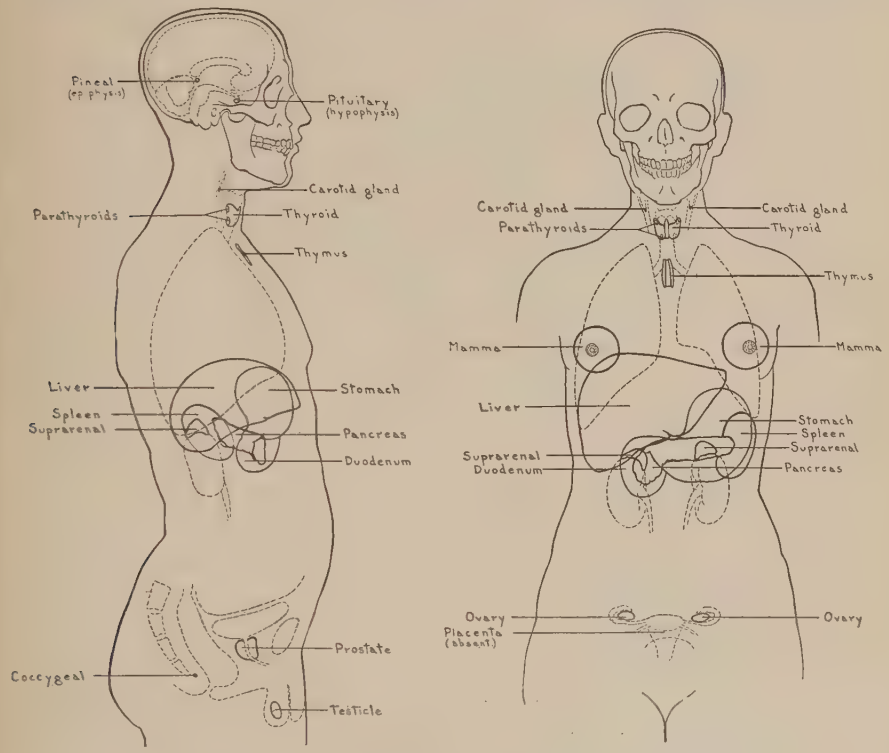


FIG. 143.—SCHEMATIC CHART OF ENDOCRINE SYSTEM.
(*From "Endocrinology and Metabolism," Appleton,* 1924.)

the real function of these glands has increased tremendously. Active extracts of two glands, pituitary extract as an ecbolic and insulin in diabetes mellitus, have a widespread clinical use. Parathyroid extract, though very recently introduced has been used for the relief of tetania parathyreopriva. In the cases of adrenaline and thyroxin the active principles have been isolated in pure form. These active principles

[1] For general references see
Endocrinology and Metabolism (Barker, Hoskins and Mosenthal) 5 vols., 1924, Appleton.
The Endocrine Organs (Sharpey-Schafer) Longmans, Green and Co., Part I, 1924; Part II, 1926.

which are manufactured in the endocrine glands and carried by the blood stream to exert an action elsewhere in the body, are called *hormones* or chemical messengers. Schafer has proposed that specific internal secretions be called *autocoids* and divides these into two classes, *hormones* which excite and *chalones* which inhibit function. A typical hormone is secretin (see page 255), formed in the mucous wall of the duodenum by the action of acid chyme on prosecretin, and carried by the blood stream to the pancreas and liver, exciting these organs to secretory activity. Acid products of muscular exertion, which stimulate respiration, are not specific regulatory substances, and have been designated[1] *parahormones*.

Hormones differ from enzymes, which are contained in the digestive secretions, etc., in that they are of more simple constitution (not proteins); they are crystallizable and dializable; they are more stable; they do not, as far as is known, require specific substrates.

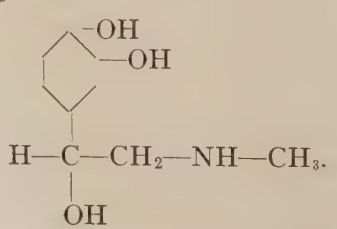
The Adrenal Glands

Degeneration of the adrenals which is known as Addison's disease was first described in 1855. The symptoms of this condition are muscular weakness, low blood pressure and bronzing of the skin. The removal of the adrenal glands in rats does not have any decided effects but when both glands are extirpated in dogs or cats the animal dies in ten days or less.

The first symptom[2] of adrenal insufficiency after extirpation is anorexia followed by diarrhea and vomiting. Muscular weakness is evident just before death. Convulsions and coma are observed in some cases. At autopsy the mucosa of the gastro-intestinal tract is frequently congested and hemorrhagic.

The adrenal or suprarenal glands are composed of two types of tissue, cortical and medullar. The cortex is thought to be essential to life although the function is unknown. It contains a high percentage of lipoids which consist mainly of lecithin and cholesterol. The medulla is characterized by its high content of adrenaline or epinephrine.

Epinephrine, which is



[1]Gley, The Internal Secretions, Translated by Maurice Fishberg, Hoeber, New York, 1917.

[2] Stewart and Rogoff: *Proc. Soc. Exp. Biol. and Med.*, 22, 394, 1924–25.

1, 2 dihydroxyphenyl, 4 hydroxyethyl-methyl amine was first isolated as the benzoyl compound by Abel[1] and as the free base by Takamine.[2] Aldrich[3] also prepared the compound and suggested the structural formula which has been accepted as correct. Stolz[4] and later Dakin[5] synthesized adrenaline.

Adrenaline may crystallize in several forms but the most common is clusters of needles. (See Fig. 144.) It is nearly insoluble in hot water, insoluble in most of the organic solvents but easily soluble in acids and alkali hydroxides. It is easily oxidized especially in alkaline solution.

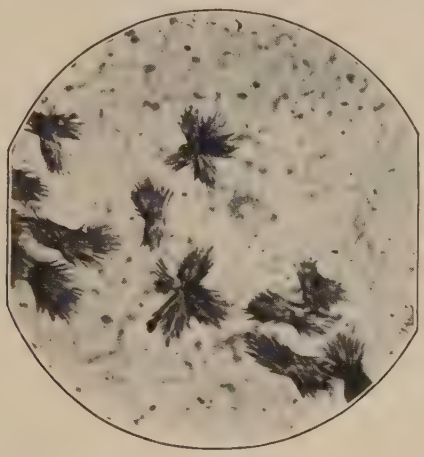


FIG. 144.—ADRENALINE (EPINEPHRINE).
(*Prepared by Arthur G. Cole, University of Illinois, College of Medicine.*)

There are two theories for the function of adrenaline, the tonus and the emergency. The tonus theory assumes that there is a constant liberation of adrenaline into the blood so that the normal arterial tone can be maintained. Stewart and Rogoff[6] state that the rate of liberation of adrenaline does not vary though the flow of blood through the glands may vary and thus change the concentration in the blood. The emergency theory which is supported by Cannon[7] states that during excitement, pain and asphysia, an increased amount of adrenaline is secreted into the blood stream so that the muscular fatigability is decreased, thus preparing the animal for an emergency.

There are two types of methods which can be used for the determination of adrenaline, the effect on the blood pressure and an

[1] Abel: *Zeit. physiol. Chem.*, 28, 318, 1899.
[2] Takamine: *Amer. Jour. Pharm.*, 73, 523, 1901.
[3] Aldrich: *Jour. Am. Chem. Soc.*, 27, 1074, 1905.
[4] Stolz: *Ber.*, 37, 4149, 1904.
[5] Dakin: *J. Physiol.*, 32, xxxiv, 1905.
[6] Stewart and Rogoff: *Am. Jour. Physiol.*, 44, 149, 1917; 52, 304, 1920.
[7] Cannon and Nice: *Am. Jour. Physiol.*, 32, 44, 1913.
Cannon: *Am. Jour. Physiol.*, 50, 399, 1919.

estimation of its reducing power. For blood pressure methods other books should be consulted.[1]

Experiments on Adrenaline

Determination of Adrenaline (Method of Folin, Cannon and Denis.)[2] Principle.—The adrenaline is extracted from the suprarenals with acid. The amount is then determined by its reducing power on the uric acid reagent of Folin and Denis.

Procedure.—The weighed glands are ground in a mortar with fine sand and N/10 hydrochloric acid. Rinse into an Erlenmeyer flask with N/10 hydrochloric acid and water using 15 c.c. of acid for each 2 grams of glands and about three times as much water. Then heat the mixture and when boiling add 5 c.c. of 10 per cent sodium acetate per 15 c.c. of hydrochloric acid. Transfer all the material to a volumetric flask (100 c.c. for each 2 grams of glands) and dilute to the mark. Either filter or centrifuge and place a 5 c.c. sample in a 100 c.c. volumetric flask. In another 100 c.c. volumetric flask place 1 c.c. uric acid solution (1 c.c. = 1 mg.) (p. 379). To each flask add 2 c.c. of Folin-Denis uric acid reagent (p. 737) and 20 c.c. of saturated sodium carbonate solution. After standing 2–3 minutes, dilute to 100 c.c. and compare in a colorimeter with the standard set as 20 mm.

Calculation.—Since adrenaline has three times the reducing power of uric acid the calculation will be:

$$\frac{20}{\text{Unknown reading}} \times 1 \times \frac{\text{Total volume}}{5} \times \frac{1}{3} = \text{mg. of adrenaline in the sample.}$$

Preparation of Adrenaline.[3]—To each 100 gm. of ground beef adrenal tissue add 50 c.c. of a 3 per cent solution of acetic acid in 95 per cent ethyl alcohol. Allow to stand for 12–24 hours and remove the liquid by straining. Re-extract the residue twice for six hours, each time with the same volume of 3 per cent acetic acid in 60 per cent ethyl alcohol as before. After filtering concentrate the extract in a flask under diminished pressure to about $\frac{1}{10}$ of the original volume. The material which has precipitated should be removed by filtration and the filtrate concentrated to about 2–3 c.c. per 100 grams of tissue extracted. Transfer the solution to a test tube and add enough strong ammonium hydroxide so that there will be a strong smell of ammonia. Stopper and set aside in a cool place for several hours. Remove the precipitate by filtration and wash first with ice-cold boiled water, then with cold alcohol and finally with ether. Dissolve the precipitate in 10 c.c. of 10 per cent hydrochloric acid, reprecipitate and treat the precipitate as before.

Properties of Adrenaline.—Dissolve 0.1 gm. of adrenaline in about 10 c.c. of 0.1 per cent HCl and dilute to 100 c.c.

(a) Vulpian Reaction—Add a few drops of $FeCl_3$ solution. A green color will be produced which is a typical catechol reaction.

[1] Stewart: "Manual of Physiology," Eighth edition, p. 210, New York, 1918. Jackson: "Experimental Pharmacology," p. 77, St. Louis, 1917.

[2] Folin, Cannon, and Denis: *Jour. Biol. Chem.*, 13, 477, 1913.

[3] From the laboratory directions of Dr. F. C. Koch, University of Chicago; Aldrich: *Am. Jour. Physiol.*, 5, 457, 1901 and Abel and Taveau: *Jour. Biol. Chem.* 1, 1, 1905.

(b) Comessatti Reaction—To 5 c.c. of the solution add an equal volume of 1 per cent sodium acetate solution and about 1 c.c. of 0.1 per cent mercuric chloride solution. A rose red color will be obtained. Warming of the solution to 40–50°C. will hasten this reaction.

(c) Test the solution with Ehrlich's Diazo Reagent (p. 674).

(d) Place a drop of the adrenaline solution on the conjunctiva of the eye of an animal. The mucous membrane will be blanched due to vasoconstriction.

(e) To demonstrate the increased blood pressure caused by adrenaline, inject subcutaneously into a guinea pig 1 mg. of adrenaline per 100 grams of body weight. Observe the animal. If the animal dies, at autopsy look for extravasation of blood into the tissues and any other effects which may be produced.

The Pancreas

The extirpation of the pancreas causes diabetes mellitus, the chief symptoms being hyperglycemia, glycosuria, and ketonuria. The body is unable to utilize carbohydrate because of the absence of the internal secretion of the pancreas. This secretion is derived from the islets of Langerhans while the digestive enzymes come from the acinar tissue.

Though extracts of the pancreas had been prepared which would reduce the blood sugar and decrease the amount of urinary glucose in a depancreatized animal, it was the work of Banting, Best, Collip and Macleod[1] which first produced an extract pure enough for administration to human diabetics. These extracts received the name *insulin* because they are derived from the islet tissue.

The first extracts were prepared by extracting the pancreas with acid alcohol and then precipitating the insulin by increasing the concentration of alcohol. This crude material can be further purified by isoelectric precipitation,[2] or precipitation of the insulin by picric acid as insulin picrate[3] which is soluble in 70 per cent acetone.

Insulin injected subcutaneously lowers the blood sugar of a normal animal. The glucose is converted into a non-saccharine material or an unrecognized form. If this lowering of the blood sugar or hypoglycemia reaches a level where convulsions are produced the overdosage of insulin can be relieved by intravenous injections of glucose. If the convulsive state has not been reached any digestible carbohydrate given by mouth will afford relief. Because insulin enables the cells of the body to metabolize glucose it may act by rendering the glucose more susceptible to oxidation, the α-β-glucose being converted into a more easily utilizable form. Lundsgaard and Holbøll[4] reported that insulin

[1] For a review of the literature consult Macleod: *Physiol. Reviews*, 4, 21, 1924. Also Dodds and Dickens: "The Chemical and Physiological Properties of Internal Secretions," London, 1925.
Jour. Metab. Res. (Insulin Number), 2, 547–985, 1922.

[2] Doisy, Somogyi, and Shaffer: *Jour. Biol. Chem.*, 55, xxxi, 1923.

[3] Dudley: *Biochem. Jour.*, 17, 376, 1923.

[4] Lundsgaard, and Holbøll: *Jour. Biol. Chem.*, 65, 305, 323, 343, 1925.

added to glucose in the presence of freshly excised muscle tissue caused the transformation of α-β-glucose into an unrecognized form because there was a decrease in the optical rotation but the decrease in the reducing power was not proportional. These observations were not confirmed by Barbour.[1]

The *unit of insulin* has been defined as $\frac{1}{3}$ the amount which will, in 5 hours, lower to the convulsive level (45 mgs. per 100 c.c.) the blood sugar of a 2 kg. rabbit which has been starved for 24 hours.

A chemical method for the standardization of insulin was suggested by Wyss[2] but the accuracy of the procedure has been questioned by Bischoff, *et al.*[3] who found that the test is not specific because proteins and amino acids have a similar action.

Insulin is destroyed by both peptic and tryptic digestion. The isoelectric point is about pH5. Its acid solution is stable and is considered to be colloidal. It is completely precipitated by tungstic, tannic and picric acids. The biuret and Pauly reactions are positive while the Molisch is negative and Millon's questionable. Most of the workers consider that insulin is protein in nature. Scott,[4] who prepared an insulin having a physiological assay of 25,000 units per gram, considers that it is similar to a protein. Funk[5] in studying the chemical composition of the insulin compound obtained with naphthol yellow S, estimates that insulin has a molecular weight of 700. This investigator considers insulin to be a polypeptide having one sulphur atom in the molecule. Abel[6] has isolated rhombohedral crystals which contained 100,000 units per gram. This crystalline material gives a positive biuret and Pauly reaction and contains labile sulphur. This labile sulphur may be the activator or catalyzer. (See page 53.)

Experiments on Insulin

1. Preparation of Insulin. Method of Fisher.[7]—To 1 kg. of finely ground fresh pancreas tissue add 1200 c.c. of 95 per cent ethyl alcohol, 300 c.c. of water, and 40 c.c. of concentrated hydrochloric acid. Allow to stand for 4–10 hours and strain through cheese cloth. Re-extract the residue with 1000 c.c. of 50–60 per cent ethyl alcohol for 4–10 hours. Then filter and combine the filtrates. After the alcohol has been evaporated by means of air blast at a temperature of not above 37°C., add 40 gm. ammonium sulphate per 100 c.c. of liquid. Separate the precipitate which rises to the top by means of a separa-

[1] Barbour: *Jour. Biol. Chem.*, 67, 53, 1926.
[2] Wyss: *Compt. rend. Acad. Sci.*, 181, 327, 1925.
[3] Bischoff, Maxwell, and Blatherwick: *Jour. Biol. Chem.*, 67, 547, 1926.
[4] Scott: *Jour. Biol. Chem.*, 65, 601, 1925.
[5] Funk, C.: *Proc. Soc. Exp. Biol. and Med.*, 23, 281, 1926.
[6] Abel: "Some Thoughts and Experiments in Relation to the Hormones." Pasteur Lecture, The Institute of Medicine of Chicago, Chicago, Jan. 22, 1926. *Proc. Nat. Acad. Sci.*, 12, 132, 1926.
[7] Fisher: *Am. Jour. Physiol.*, 67, 57, 1923.

tory funnel and discard the liquid portion. This precipitate should be ground with two separate 75 c.c. portions of 70 per cent alcohol and filtered. To the filtrate add an equal volume of 95 per cent alcohol which will precipitate a small amount of insulin plus some toxic material which causes sterile abscesses on subcutaneous injection. After removing the precipitate add 8 volumes of 95 per cent ethyl alcohol. Remove the resulting precipitate of insulin by filtration. Dissolve the precipitate in water using 1 c.c. of water for each 10 grams of pancreas tissue. Preserve with 0.1 per cent tricresol and keep in an ice box.

The yield of insulin will vary from 0.5 to 3 units per gram of tissue depending on the freshness of the glands and the care in preparation.

2. Effect of Insulin on the Blood Sugar.—Take a 1 c.c. sample of blood for a sugar determination from the marginal ear vein of a 2 kg. rabbit which has been starved for 24 hours. Then inject subcutaneously 5 to 10 units of insulin. Observe the rabbit carefully for symptoms of hypoglycemia, such as hyperirritability, palpitation of the heart, convulsions and coma. Another sample of blood should be drawn as soon as distress is evident and a third when the rabbit is in convulsions. To relieve the induced hypoglycemia inject 10 c.c. of 10 per cent glucose solution intravenously.[1] After recovery take another blood sample for a sugar determination. Compare the blood sugar values.

The Thyroid Gland[2]

If the thyroid gland[3] is removed or fails to develop sufficiently in a young animal a cretin is produced. (See Fig. 145.) The growth and mental development are both retarded. When the thyroid is extirpated or produces an insufficient secretion in an adult myxedema results. (See Fig. 146.) The skin is thick and dry, the metabolic rate is lowered and there is mental deterioration. The condition is sometimes called cachexia strumipriva. In overactivity or hypersecretion there is an increased metabolic rate, nervousness, exophthalmos and tachycardia. Baumann[4] in 1895 reported that the thyroid contained appreciable amounts of iodine. Later Ostwald[5] prepared the iodine complex of the thyroid gland which he called iodo-thyreoglobulin. Koch[6] found that the thyreoglobulin and the metaprotein derived by hydrolysis contained the active principle of the thyroid gland as judged by the acetonitrile test for thyroid. The primary albumose, iodothyrin and secondary albumose had a very low activity and the amino acid fractions had negligible activity.

[1] If desired the hypoglycemia can also be relieved by feeding the rabbit some glucose on a leaf of lettuce or cabbage or by giving glucose by stomach tube.

[2] For a review of the literature consult Scharpey-Schafer, E.: "The Endocrine Organs." Second edition, London, 1924, Part one, page 14.

[3] At least one parathyroid must be left intact or tetania parathyreopriva will supervene.

[4] Baumann: *Zeit. physiol. Chem.*, 21, 319, 1895.

[5] Ostwald: *Arch. Exp. Path. u. Pharm.*, 60, 115, 1909 and 63, 263, 1910.

[6] Koch: *Jour. Biol. Chem.*, 14, 101, 1913.

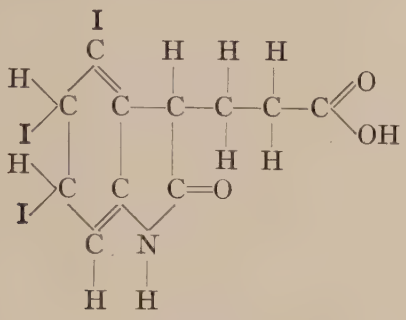
FIG. 145.—EFFECT OF THYROXIN ON GROWTH OF A CRETIN.

The illustration shows the same child, in the same dress, before and after receiving thyroxin for one year. Increase in height 6 inches. (*From Kendall: Ind. Eng. Chem.*, 17, 525, 1925.)

FIG. 146.—EFFECT OF THYROXIN ON MYXEDEMA.

The time interval between pictures is 3 weeks. The total amount of thyroxin used was less than 20 mg. (*From Kendall: Ind. Eng. Chem.*, 17, 525, 1925.)

Kendall[1] isolated a crystalline substance (See Fig 147) from the thyroid gland which he called *thyroxin*, $C_{11}H_{10}O_3NI_3$, and to which he assigned the following structural formula:

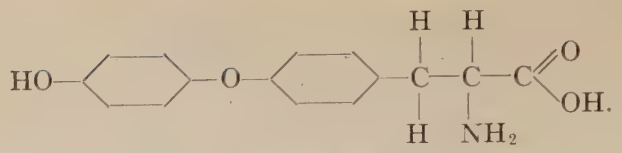


Thyroxin
4, 5, 6, tri-iodo-5, 6,-dihydro-2-oxyindol-propionic acid.

FIG. 147.—ONE OF THE CRYSTAL FORMS IN WHICH THYROXIN SEPARATES FROM AN ALCOHOL SOLUTION.
(*From Kendall: Ind. Eng. Chem.*, 17, 525, 1925.)

This formula has been questioned by Harington.[2] He has prepared thyroxin by a simpler method which gives a much better yield than Kendall's process. The empirical formula proposed is $C_{15}H_{11}O_4NI_4$. When the iodine was removed by treatment with hydrogen using palladium hydroxide-calcium carbonate as a catalyst, *desiodothyroxin*

[1] Kendall: *Ind. and Eng. Chem.*, 17, 525, 1925; Kendall and Osterberg: *Jour. Biol. Chem.*, 40, 265, 1919.
[2] Harington: *Biochem. Jour.*, 20, 293 and 300, 1926.



Desiodothyroxin

or more simply the *p*-hydroxyphenyl ether of tyrosine was produced. This compound has been synthesized by two different methods. From experimental work which is still incomplete, it is indicated that the iodine atoms occupy the 3, 5, 3′ and 5′ positions.

Thyroxin is a white, odorless and tasteless substance. It is not soluble in any of the organic solvents which are not acid or basic in nature. Sodium, potassium and ammonium hydroxides dissolve thyroxin but it is insoluble in solutions of the carbonates of these metals.

The amount of this material required per day is about 2 mg. The entire physiological activity of the thyroid gland is due to the thyroxin. This has been proven by experimental tests on tadpoles, the acetonitrile test and the effect on the basal metabolic rate.

Experiments on the Thyroid

Preparation of Iodo-thyreoglobulin. Method of Nürenberg.[1]—Either the fresh or dried thyroid glands may be used. The glandular material is extracted three or four times with ten times its volume of 0.8 per cent sodium chloride solution. The globulin is precipitated by the addition of an equal volume of saturated ammonium sulphate. After filtration the precipitate should be redissolved in dilute salt solution and reprecipitated. This process should be repeated once. Then dissolve the precipitate as before and dialyze until free from salt and finally precipitate by adding twice the volume of 95 per cent ethyl alcohol. After filtering, dry this precipitate of thyreoglobulin. This material contains all of the active principle of the thyroid gland.

Determination of the Activity of Thyroid Products. Method of Hunt.[2]—Principle.—The susceptibility of mice to acetonitrile poisoning is decreased by thyroid feeding.

Procedure.—Feed four or more experimental mice for four days with cracker dust made into tablets or pellets of about 2–3 grams weight. Then divide the mice into four groups and feed as follows for 10 days: Group 1. Control. Keep on a cracker diet. To the other three groups incorporate thyroid material as follows into the cracker pellets. Group 2. 1 mg. of dried thyroid. Group 3. 0.25 mg. iodo-thyreoglobulin, and Group 4, 0.003 mg. thyroxin.[3] Notice the loss of weight of the mice which have received the thyroid material Then

[1] Nürenberg: *Biochem. Zeit.*, 16, 87, 1909.

[2] Hunt: *Jour. Biol. Chem.*, 1, 33, 1905; *Am. Jour. Physiol.*, 63, 257, 1922. For another method of showing the activity of thyroid material consult Mark: *Pflüger's Archiv.*, 209, 437, 1925.

[3] Thyroxin can be obtained from E. R. Squibb and Sons, New York.

inject 0.4 mg. of acetonitrile per gram body weight into the mice which have not been fed thyroid material and 1.2 mg. per gram of body weight into the mice which have been fed the thyroid material. The untreated mice will usually die in less than 2 hours while the treated mice, though receiving 3 times as much acetonitrile, live.

Determination of Iodine in Thyroid Tissue. Method of Kendall.[1] **Principle.**—The organic matter is destroyed by NaOH and KNO_3, the iodine being retained as NaI. After liberation the iodine is titrated with sodium thiosulphate.

Procedure.—Use a sample not larger than 0.5 gram. Place the material in a 5.9 cm. nickel crucible, moisten with a few drops of 30 per cent sodium hydroxide, and add 5–10 grams of solid sodium hydroxide which has been broken into small pieces. The excess water should be evaporated on a hot plate. The crucible is then heated indirectly by placing it inside an iron crucible, 7.8 cm. in diameter, the bottom of which is covered with a layer of sand 0.5 cm. in thickness. The larger crucible is supported inside an iron cylinder 9.4 cm. in diameter having cross bars which support the crucible 22.5 cm. from bottom. For heating a No. 3 Meker burner is preferred. The larger crucible should be heated to a red heat. After the melt has settled to the bottom and very few bubbles of gas are being liberated, remove the smaller crucible and agitate the contents by rotatory motion. Then add 5–10 mg. of potassium nitrate to oxidize the remaining organic matter. Repeated additions of 5–10 mg. of the nitrate are made until there are no more bubbles of gas liberated. Then pour the melt into the cover of the crucible and allow it to cool.

After cooling, dissolve the melt in 125–150 c.c. of water and transfer to a 500 c.c. Erlenmeyer flask. The volume should be about 200 c.c. Then add 5 c.c. of 20 per cent sodium bisulphite solution and 2 drops of a saturated water solution of methyl orange. After cooling add 85 per cent phosphoric acid till the indicator turns pink. Add a piece of hard coal 0.5 cm. in diameter and 5 c.c. of 20 per cent phosphoric acid. Adjust to a volume of 250 c.c. and boil on a hot plate for 10 minutes or longer until the volume is about 200 c.c. Allow the flask to cool and add sufficient bromine so that a distinct yellow color is produced. Place the flask again on the hot plate and note the time when all the visible bromine has been expelled. Then boil for 5 minutes more. Remove from the hot plate, add a few crystals of salicylic acid and immerse the flask in cold water. The total volume should not be less than 175 c.c. as too high a concentration of salts makes the end point indistinct. After adding 5 c.c. of 20 per cent reduced phosphoric acid[2] and about 1 gm. of pure potassium iodide, titrate the liberated iodine with 0.005 N sodium thiosulphate[3] using starch as an indicator in finishing the titration.

Determination of Iodine in Blood and in Animal Tissues. Method of Kendall and Richardson.[4] **Principle.**—The organic matter is destroyed by NaOH and KNO_3, the iodine being retained as NaI. After liberation the iodine is titrated with sodium thiosulphate.

[1] Kendall: *Jour. Biol. Chem.*, 19, 251, 1914; 43, 149, 1920. For another method consult Pickworth: *Biochem. J.*, 19, 768, 1925.

[2] Any oxidizing action of the phosphoric acid would liberate free iodine. To destroy the oxidizing action, dilute the 85 per cent phosphoric acid with 4 volumes of water and boil with strips of aluminum. The reduced acid should give no color in 15 minutes when 25 c.c. is added to 1 gm. of potassium iodide using starch as the indicator. It is unnecessary to reduce the phosphoric acid which is used for neutralization.

[3] For preparation and standardization see Reagents and Solutions (Appendix).

[4] Kendall and Richardson: *Jour. Biol. Chem.*, 43, 161, 1920.

Procedure.—Place 100 gm. of tissue or 100 c.c. of blood in a 500 c.c. nickel crucible, add 10 c.c. of 30 per cent sodium hydroxide and mix the material so that it will be homogeneous. After evaporating the water on a hot plate, the crucible is placed in a metal cylinder having a metal disc support which is covered with an asbestos pad. A Meker burner should be adjusted so that all the fumes will be evolved in 12 minutes. Cover the crucible after contents have subsided (6–7 minutes). Allow the crucible to cool after no more fumes are given off. Then add 25 c.c. of water, scrape off the charred material inside the crucible and grind with a pestle. Boil after adding 25 c.c. more of water and remove the carbon by filtration on a Buchner funnel. Return the carbon with the filter paper to crucible and boil again with 30 c.c. of water containing 1–2 c.c. of 50 per cent sulphuric acid. Filter on a Buchner funnel and combine the filtrates. To this solution add 18 gm. of crystalline barium hydroxide[1] and filter through the same Buchner funnel (char still present). Add enough sulphuric acid to remove all of the barium and filter through a pad of kaolin on a Buchner funnel. Place the solution on a hot plate, add a small piece of hard coal and evaporate to a volume of 15–20 c.c. Transfer to a 100 c.c. nickel crucible and evaporate the last traces of water in a hot air chamber in which a rapid current of air from an inverted funnel is blown over the surface of the crucible. Then add 4–5 gm. of solid sodium hydroxide and proceed as in the method for iodine in the thyroid gland. As little potassium nitrate as possible should be added, not over 4–5 mg. at any one time. If the solution of the fusion melt is turbid it should be filtered through a layer of kaolin in a suction funnel. Frequent tests for iodine on the materials used must be made. The funnels and flasks should not be used for other work. Iodine should not be used in the same room.

The Ovaries[2]

The ovaries maintain the sex life of the female by hormone mechanisms. The liquor folliculi of the ovary secretes a specific chemical substance into the blood which induces the estrus cycle. The presence of this female sex hormone has been demonstrated in the circulating blood of an animal in the estrus cycle.[3] This hormone which induces estrus in an ovariectomized rat has been prepared from the liquor folliculi. Doisy *et al.*[4] treat the liquor folliculi with twice the volume of alcohol which precipitates the proteins. The material is then purified by the use of organic solvents. Whole ovaries or placenta can also be used in the preparation of this hormone.

The activity of the preparations is judged by the smear method of Stockard and Papanicolaou.[5] The vaginal smear of a castrated rat

[1] The barium hydroxide should be recrystallized twice from hot water. It should be tested for iodine by precipitating 100 gm. with carbon dioxide after adding 3 gm. of sodium hydroxide. Remove the barium carbonate by filtration, neutralize the sodium carbonate with phosphoric acid and carry out the regular method for the estimation of iodine.

[2] For reviews consult Novak: *Endocrinol.*, 6, 599, 1922. Frank: *Jour. Am. Med. Assoc.*, 78, 181, 1922. Carlson: *Jour. Am. Med. Assoc.*, 83, 1920, 1924.

[3] Frank, Frank, Gustavson and Weyerts: *Jour. Am. Med.* Assn., 85, 510, 1925 and Frank and Goldberger: *ibid.*, 86, 1686, 1926.

[4] Doisy, Ralls, Allen, and Johnston: *Jour. Biol. Chem.*, 61, 711, 1924. Frank and Gustavson: *Jour. Am. Med. Assn.*, 84, 1715, 1925. A more recent method has been described by Ralls, Jordan and Doisy: *Jour. Biol. Chem.*, 69, 357, 1926.

[5] Stockard and Papanicolaou: *Am. Jour. Anat.*, 22, 225, 1917.

consists mainly of leucocytes while the smear during the estrus cycle contains squamous, non-nucleated epithelium. These extracts cause, in a spayed animal the typical estrual hyperemia[1] and thickening of the vaginal walls. Several injections will cause premature puberty in a young animal. A *rat unit* is defined as the quantity of material necessary to induce estrus in an ovariectomized sexually mature rat weighing 120–160 gms. As small an amount as .04 mg. of material prepared by Doisy, *et al.*[2] contained one rat unit.

This hormone of the liquor folliculi is soluble in the organic solvents such as ether, chloroform, alcohol and acetone. It is thermostable and not an ester or fatty acid. The compound is composed of carbon, hydrogen and probably oxygen, is unsaturated and does not contain a carbonyl group.

The demonstration of the female sex hormone in the blood may be used to establish the sex of a person in whom the external genitals are malformed and the internal organs of generation are malformed or absent.[3]

The corpus luteum of the ovary has an inhibitory action on ovulation and estrus since its removal abolishes these phenomena. An extract capable of producing this inhibitory action when injected parenterally has been prepared from the corpus luteum by Papanicolaou.[4] The oral administration is ineffective.

Experiments on the Ovary

Preparation of an Ovarian Hormone.[5] Procedure.—Aspirate the liquor folliculi from hog ovaries, or use the finely ground whole ovaries or placenta for the preparation. Coagulate the protein by adding at least two volumes of 95 per cent alcohol. After filtering off the proteins, they are extracted for 6 hours in a Soxhlet extractor with 95 per cent ethyl alcohol. The filtrate and extract are combined and evaporated to dryness in a current of warm air. The residue after emulsification in a small amount of water is transferred to a flask. Enough 95 per cent ethyl alcohol is added to make the total volume 15 c.c. per 100 c.c. of liquor folliculi. After heating the alcohol to boiling add 2 volumes of acetone. Cool and filter. Repeat the alcohol-acetone treatment three times with the residue. Evaporate the combined filtrates to dryness and leach the residue 5–7 times with small volumes of boiling 95 per cent alcohol with subsequent cooling in a salt-ice mixture before filtration. Distill off the alcohol and take up the residue in anhydrous ether. Remove the precipitate from ether by centrifuging and keep this solution as the stock supply. When ready for use, evap-

[1] Similar observations on primates (monkeys and human beings) have been made by Pratt and Allen: *Jour. Amer. Med. Assn.*, 86, 1964, 1926.

[2] Ralls, Jordan, Hewsinkveld, and Doisy: *Jour. Biol. Chem.*, 67, v, 1926.

[3] Frank and Goldberger: *Jour. Am. Med. Assn.*, 87, 554, 1926.

[4] Papanicolaou: *Proc. Soc. Exp. Biol. and Med.*, 22, 106, 1924–25. *Jour. Amer. Med. Assn.*,86, 1422, 1926.

[5] Doisy, Ralls, Allen and Johnston: *Loc. cit.*

orate the ether and dissolve in purified corn oil for injection. The yield from liquor folliculi varies from 220–2,180 rat units per kg. of liquid. Whole ovaries yield 80–200 rat units per kg. of tissue and from human placenta 400–700 units per kg. may be obtained.

The Action of the Ovarian Hormone.[1]—Ovariectomize an adult rat. After 4–6 days the vaginal smear will consist mainly of polynuclear leucocytes. Then inject three subcutaneous injections of 1 c.c. each of the hormone in oil. The second day the vaginal smear will be composed chiefly of nucleated epithelial cells. The third day's smear will consist of non-nucleated epithelial cells which stain with eosin. This type of cell is a positive test as it is present in a typical estrus smear.

To show the effect of the hormone on the uterus, inject about three units into an 8 week old female rabbit keeping a litter sister for a control. Continue the injections for about 8 days. Kill both rabbits and compare the size of the Fallopian tubes and the uterus. The genital system of the injected rabbit will be larger in size and may be hyperemic.

The Testes

The development of the secondary sexual characters of the male is dependent on the internal secretion of the testes. This hormone is supposedly elaborated by the interstitial cells. No active extract or principles have been extracted. Ligation of the vas deferens which may result in a subsequent interstitial cell hypertrophy does not cause rejuvenescence.[2] Castration lowers the metabolism of the body. The voluntary activity of white rats is greatly reduced by castration.[3] Emulsions of the testes and extracts thereof have only a slight effect on the nitrogen metabolism of rabbits.[4]

The Pituitary Gland[5]

The pituitary gland consists of two lobes, an anterior and a posterior. The literature is voluminous but it is very conflicting due to the difficulties in surgical removal. The syndrome produced by removal as well as that associated with dysfunction are not well understood. The results of feeding the gland are contradictory. In Fröhlich's syndrome, which results if the anterior lobe is extirpated or decreases in function before puberty, the sexual organs remain infantile, the stature remains small and there is marked adiposity. This view has been questioned by Dandy and Reichert.[6] Acromegaly and gigantism are both attrib-

[1] Allen, *et al*: *Am. Jour. Anat.*, 34, 133, 1924.
[2] Oslund: *Science*, 60, 419, 1924.
[3] Hoskins: *Am. Jour. Physiol.*, 72, 324, 1925.
[4] Korenchevsky and Carr: *Biochem. Jour.*, 19, 773, 1925.
[5] Bell: "The Pituitary," New York, 1919.
Geiling: *Physiol. Reviews*, 6, 62, 1926.
[6] Dandy and Reichert: *Bull. Johns Hopkins Hosp.*, 37, 1, 1925.

uted to hypersecretion of the anterior lobe. Robertson[1] prepared a principle from the anterior lobes which he called *tethelin*. This material on oral administration increased the growth and duration of life in mice. Drummond and Cannan[2] failed to confirm Robertson's results. Evans and Long[3] found no increase in growth or any effect on the estrus cycle of rats by the oral administration of either fresh or dried anterior lobes of cattle, but when an extract was administered intraperitoneally there was a decided increase in growth and a cessation of the estrus cycle. These extracts were prepared from freshly dissected anterior lobes of the ox. The lobes were first washed by placing them for five minutes in 40 per cent ethyl alcohol and later rinsed with two portions of saline solution. After grinding in a mortar the material was treated with two volumes of saline solution and centrifuged for one-half hour. One c.c. of the supernatant liquid represented about two grams of the beef anterior lobe. The injections which were made intraperitoneally began on the fourteenth day of life when the young were still sucklings. At the start only one-eighth c.c. was used and eventually increased to 1–1.5 c.c. The rats which were injected increased in weight much more rapidly than the controls, the weight increase being about 50 per cent in 200 days. This increase in weight was not due to fat but was a true overgrowth except for the reproductive tract which remained infantile. Estrus was infrequent or completely absent.

The posterior lobe may be concerned in diabetes insipidus. The subcutaneous injection of extracts of the posterior lobe will relieve the condition. A highly active tartrate preparation produced by Abel and Geiling[4] has also alleviated this abnormal state. However, it has been found that superficial lesions of the base of the brain cause diabetes insipidus.[5] Water or dilute acid extracts of this lobe have a pressor effect and also a decided oxytocic action. The active principle is destroyed by peptic and tryptic digestion, is soluble in hot alcohol, ethyl butyrate, dilute acids, water and is insoluble in chloroform, carbon disulphide and petroleum ether. The active principle or principles have not been separated in a pure form but they have shown a very high degree of activity.[6] This material will cause a contraction of the isolated horn of the uterus of a virgin guinea pig in a dilution of 1:18,750,000,000.

[1] Robertson: *Jour. Biol. Chem.*, 24, 385, 1916.
[2] Drummond and Cannan: *Biochem. Jour.*, 16, 53, 1922.
[3] Evans and Long: *Anat. Record*, 21, 62, 1921. Evans, H. M.: The Harvey Lectures, New York, 1923–24.
[4] Abel and Geiling: *J. Pharm. and Exper. Ther.*, 22, 317, 1923.
[5] Camus and Roussy: *Endocrinol.*, 4, 507, 1920. Bourquin: *Am. Jour. Physiol.*, 76 181, 1926.
[6] Abel, Rouiller, and Geiling: *Jour. Pharm. and Exper. Ther.*, 22, 289, 1923.

The Thymus

The function of the thymus in most mammals is not well understood. Park and McClure[1] who extirpated the thymus gland in puppies reported that there was no alteration in hair, teeth, strength, activity or intelligence. Thymectomy in young, adult or old rabbits does not have any appreciable effect.[2] In pigeons, Riddle[3] found that the birds with small thymi were producing eggs in which the shells were soft. Oral administration of ox thymus corrected the disorder. The thymus gland influences the oviduct so that a normal egg-envelope is produced. The name suggested for this hormone is *thymovidin*.

The Pineal Gland[4]

Although the pineal may be a vestigial remnant its dysfunction has been cited as the cause of numerous somatic disturbances. From the results of extirpation by Dandy[5] it would seem that this gland does not have any endocrine function in either young or old dogs.

The Parathyroids[6]

If the parathyroid glands are extirpated in a dog kept on a meat diet, parathyroid tetany usually ensues in 24–72 hours after the operation. The first symptoms are clonic contractions or tremors of the skeletal muscles followed by hyperpnoea and marked salivation with a decided spasticity of the skeletal musculature. There may be a spasm of the larynx as well. The attacks usually last 2–4 hours and the animals as a general rule die in the first attack.

There are two theories to explain this type of tetany. The first states that the glands act as a detoxicating mechanism for the guanidine $\begin{matrix} \diagup NH_2 \\ C \quad NH \\ \diagdown NH_2 \end{matrix}$, guanidine derivatives and other toxins which can be absorbed from the intestine. Guanidine and some of its derivatives like methyl and dimethyl guanidine have been isolated from the urine of parathyroidectomized dogs by Koch[7] and also by Paton, Findlay, *et al.*[8] Greenwald[9] has failed to demonstrate the presence of any toxic material

[1] Park and McClure: *Am. Jour. Dis. Child.*, 18, 317, 1919.
[2] Van Allen: *Jour. Exp. Med.*, 43, 119, 1926.
[3] Riddle: *Am. Jour. Physiol.*, 68, 557, 1924.
[4] For a review consult McChord: "Endocrinology and Metabolism." Vol. 2, p. 7. Appleton, New York and London, 1924.
[5] Dandy: *Jour. Exper. Med.*, 22, 237, 1915.
[6] For a review of the subject see Collip: *Medicine*, 5, 1, 1926.
[7] Koch: *Jour. Biol. Chem.*, 15, 43, 1913.
[8] Paton, Findlay, *et al: Quart. Jour. Exper. Physiol.*, 10, 203, 1916.
[9] Greenwald,: *Jour. Biol. Chem.*, 61, 33, 1924.

in the blood of parathyroidectomized dogs. Guanidine does not seem to be the cause of parathyroid tetany because Collip and Clark[1] found that there was not an increase of the urea and non-protein nitrogen in the blood of a parathyroidectomized dog until the terminal stage of tetany, while after the administration of guanidine there was a gradual increase of both the urea and the non-protein nitrogen of the blood. Dragstedt and Sudan[2] have shown that guanidine intoxication produces no marked hypocalcemia in thyro-parathyroidectomized dogs that had been kept free from tetany by oral administration of calcium lactate or kaolin.[3]

The second theory of parathyroid tetany is based on the fact that the calcium of the blood serum is low, usually below 7 mgs. per 100 c.c. of serum preceding and during an attack. This fact was first noted by MacCallum and Vogel.[4]

Salvesen[5] claims that the tetany is due to low blood calcium and that the function of the parathyroids is to regulate the blood calcium. This has been confirmed by Collip and his collaborators.[6] There is some evidence[7] that the calcium may act by inhibiting the breaking down of the lactacidogen of the muscles and in this way prevent the spasticity of the muscles which is so pronounced in tetany.

This type of tetany can be controlled by various measures; a milk, bread and lactose diet,[8] by intravenous injections of Ringer's solution or calcium lactate[9] and by the use of extracts prepared from the parathyroid glands (Collip,[10] Hansen[11] and Fisher and Larson[12]). These extracts raise the level of the blood calcium as well as control the tetany. The methods of preparation are all dependent on an acid extraction of ox parathyroids and a subsequent purification by removal of most of the proteins. The extracts prepared by Collip do not prevent the tetany which can be produced by guanidine injections. The *unit of parathyroid extract* is 1/100 of the amount which will produce an average increase of 5 mg. in the blood serum calcium of normal dogs weighing 20 kg., in a period of 15 hours. Since Vines[13] proposed a method of standardizing parathyroid preparations by the removal of guanidine

[1] Collip and Clark: *Jour. Biol. Chem.*, 67, 679, 1926.
[2] Dragstedt and Sudan: *Am. Jour. Physiol.*, 77, 321, 1926.
[3] Dragstedt and Sudan: *Ibid.*, 77, 296, 307, 314, 1926.
[4] MacCallum and Vogel: *Jour. Exper. Med.*, 18, 618, 1913.
[5] Salvesen: *Jour. Biol. Chem.*, 56, 443, 1923.
[6] Collip *et al.*: *Jour. Biol. Chem.*, 63, 439, 395, 1925; 64, 485, 1925.
[7] Martin, *Quart. Jour. Med.*, 19, 311, 1926.
[8] Dragstedt *et al.*: *Am. Jour. Physiol.*, 65, 368, 503, 1923.
[9] Luckhardt *et al.*: *Science* 56, 48, 1922, *Jour. Am. Med. Assoc.*, 80, 79, 1923.
[10] Collip *et al.*: *Jour. Biol. Chem.*, 63, 395, 439, 1925; 64, 485, 1925.
[11] Hanson: *Military Surgeon*, 55, 701, 1924.
[12] Fisher and Larson: *Am. Jour. Physiol.*, 75, 93, 1925.
[13] Vines: *Brit. Med. Jour.*, 2, 559, 1923.

from a solution. White and Cameron[1] applied this method to Collip's active extract. They question the accuracy of Vine's method of testing.

Experiments on the Parathyroids

Preparation of an Active Parathyroid Extract. Method of Collip.[2] Place the fresh ox parathyroids, from which the visible fat and connective tissue has been removed, in a small flask or test tube, cover with an equal volume of 5 per cent hydrochloric acid and keep in a boiling water bath for one hour. Allow to cool and remove the fat mechanically. Then make the solution faintly alkaline with sodium hydroxide and add acid to the point of maximum precipitation of protein which is just barely acid to litmus. Filter and dissolve the precipitate in weak alkali and reprecipitate as before. Filter again and combine the filtrates adding 0.1 per cent tricresol as a preservative and keep in an ice box till ready for use.

Effect of Parathyroid Extract.[3]—Draw a blood sample of 8–10 c.c. from either the saphenous vein or directly from the heart of a dog. Save this for a calcium determination by the Clark-Collip modification of the Tisdall method (p. 408). Inject subcutaneously into the dog the extract from 5 glands per 10 kilos of body weight every hour for six hours. Draw blood samples for calcium determination as before at end of 10, 15, 25 and 40 hours. Note the effects of the induced hypercalcemia such as depression, atonia and anorexia. The blood will be concentrated during the period of hypercalcemia. Compare the calcium values.

[1] White and Cameron: *Trans. Royal Soc. Canadian*, Section V, 45, 1925.
[2] Collip: *Jour. Biol. Chem.*, 63, 395, 1925.
See also Fisher and Larson: *Am. Jour. Physiol.*, 75, 93, 1925.
[3] Parathyroid extract (Para-Thor-mone) may be obtained from Eli Lilly and Co., Indianapolis, Indiana.

CHAPTER XXI

ENERGY METABOLISM

Combustion in living beings was first described by Lavoisier (1743–94). This brilliant chemist discovered the true nature of oxygen, and how its union in the body with carbon and hydrogen resulted in the formation of carbon dioxide and water and in the production of heat. In experiments on man he determined that oxidation was increased by food, by a cold environment, and by muscular work. He knew the main constituents of the atmosphere and that nitrogen played no active part in animal respiration. Lavoisier was under the false impression that oxygen decomposed some fluid in the lung causing the liberation of hydrogen and carbon with the subsequent oxidation of these elements and their excretion as carbon dioxide and water in the expired air. Scientists of a later date abandoned the theory of oxidation in the lungs and favored the blood as the site of these chemical changes. This belief was strengthened by the discovery of gases in the blood by Magnus in 1837. Later it became known that for the most part, oxidation occurred in the tissues and that the blood simply carried the gases to and from the lungs.

In 1842 Liebig announced that the substances burned in the body were carbohydrate, fat, and protein and suggested that the urinary nitrogen would serve as an index of the extent of protein destruction. Ten years later Bidder and Schmidt described protein metabolism and shortly afterwards Carl Voit measured protein waste by determining the amount of nitrogen in the excreta. Voit made the important discovery that muscular exercise did not increase protein metabolism. During many years of careful work Rubner, a pupil of Voit, determined the fuel values of the foodstuffs and by means of a respiration calorimeter showed that the heat production as calculated from the respiratory exchange was the same as that obtained by the direct measurement of the heat given off by the body. In 1883 Rubner demonstrated the relationship between the surface area of the body and the heat production, thus providing a basis for comparison of the metabolism of different individuals.

In 1915 DuBois devised the most satisfactory method for estimating the surface area of the body and later published normal standards of

heat production for males and females, the accuracy of which has been fairly well established.

In America, Atwater, Armsby, Benedict, Lusk, DuBois and their many associates have added valuable contributions to the knowledge of normal metabolism.[1] Benedict and those associated with him in the Carnegie Nutrition Laboratory have in addition perfected various forms of respiration apparatus, and they are chiefly responsible for the extensive use of calorimetry in the clinic.

Physical and Chemical Bases of Animal Calorimetry

Normal Temperature is 0° Centigrade or 273° of absolute temperature.

Normal Pressure is 760 mm. of mercury at 0°C.[2]

Gases.—Gases are compressible fluids. The molecules of which they are composed are under constant motion and tend to disperse. The gas therefore *expands* and fills all parts of the containing vessel and exerts *pressure* upon the enclosing walls. Heat increases the tendency to expand and so increases the pressure unless the container permits the gas to assume a larger *volume.* If the temperature remains constant and pressure is applied to the container, the molecules are forced closer together and the volume is decreased. Therefore, when we speak of a volume of gas we must also state the temperature and pressure under which it was measured. Gas volumes are always recorded as their volume at normal temperature and normal pressure.

If the temperature is kept constant, the volume of a gas varies inversely with the pressure. This law was discovered by Robert Boyle in 1662.

To express a volume of gas at 760 mm. the formula is:

$$\text{Volume} \times \frac{\text{Pressure (corrected)}}{760}$$

[1] The following are some important contributions to the literature on normal and pathological metabolism:

Atwater and associates: Bulletins of U. S. Dept. of Agriculture (Office of Experiment Stations) and Reports of Storrs Agric. Exp. Sta., 1887, *et seq.*

Atwater and Benedict: Carnegie Inst. Wash. Pub. No. 42, 1905.

Armsby and Fries: Bulletin 51, U. S. Dept. of Agriculture (Bureau of Animal Industry), 1903.

Armsby, Fries and Braman: *Jour. Agric. Res.*, 13, 43, 1918.

Benedict and associates: Publications of Carnegie Inst. of Wash., 1905, *et seq.*

Lusk: The Science of Nutrition, Philadelphia, Saunders, 1917.

DuBois: Basal Metabolism in Health and Disease, Philadelphia, Lea and Febiger, 1924.

Endocrinology and Metabolism, New York, Appleton and Co., 1924.

Boothby and Sandiford: *Physiol. Rev.* 4, 69, 1924.

McCann: *Medicine*, 3, 1, 1924.

Carpenter: (Tables, factors, formulæ, etc.) Carnegie Inst. Wash., Pub. No. 303, 1921.

Krogh: Respiratory Exchange of Animals and Man, London, Longmans Green, 1926.

[2] The reading on a brass scale barometer is correct for only one temperature. The reading must be corrected by the use of a correction table (see Appendix) which gives the height at which the column of mercury would stand if the temperature were 0°C.

Law of Charles (1746–1823) or *Gay-Lussac* (1778–1850). If the pressure on a gas remains the same and the temperature changes, the volume of the gas will vary directly $\frac{1}{273}$ (or 0.00367) of its volume at 0°C. for each degree of change in temperature. The coefficient of expansion of nearly all gases is 0.00367.

To express a volume of gas at 0°C. the formula is:

$$\text{Volume} \times \frac{273}{273 + \text{t°C.}} = \text{Volume} \times \frac{1}{1 + 0.00367\text{t°C}}.$$

Law of Avogadro (1776–1856). Equal volumes of different gases or vapors under like conditions of temperature and pressure contain the same number of molecules.

Density of Gases.—From the above law it is evident that the density or *mass per volume* of any gas or vapor will depend upon the weight of its molecule. The molecular weight in grams of any gas or vapor has a volume of practically 22.4 liters at 0°C. and 760 mm. Hence the density of 1 liter of a gas at 0°C. and 760 mm. is: molecular weight ÷ 22.4.

Density of gases at 0°C. and 760 mm.

1 liter of oxygen weighs 1.4292 gram.

1 gram of oxygen occupies $\frac{1}{1.4292} = 0.6997$ liter.

1 liter of carbon dioxide weighs 1.9652 gram.

1 gram of carbon dioxide occupies $\frac{1}{1.9652} = 0.5089$ liter.

Evaporation in Air.—If a volume of air is in contact with water, evaporation continues until the tendency of the water to evaporate is equal to the tendency of the vapor to condense, at which time the air is said to be *saturated* with water vapor. The total pressure of the air and water vapor is equal to the sum of the pressures of the air and of the water vapor. These are spoken of as *partial pressures*. The partial pressure of the water vapor increases with temperature. To determine the pressure of the air in a dry state, the partial pressure of water vapor at the given temperature must be deducted from the barometric pressure. (See Appendix.)

Example.—The expired air of a man has a volume of 100 liters, the temperature in the gasometer is 22°C., and the brass scale barometer reads 767 mm.

The pressure of expired air, *dry*, would be:

767 mm. − 2.76 (barometer correction) − 19.84 (water vapor) = 744.4 mm.

The volume of dry air at 0°C. and 760 mm. would be:

$$100 \times \frac{744.4}{760} \times \frac{1}{1 + 0.00367 \times 22} = 90.65 \text{ liters}$$

Heat.—The heat which we recognize by *temperature* is the energy of molecular motion. This property is imparted to matter by chemical action, electric currents, and mechanical work. In animals the chemical source only need be considered and we may confine our attention to that form of combustion in which oxygen unites with the burning substances.

In animal calorimetry the unit of heat is the *large calorie*, or the amount of heat necessary to raise the temperature of 1 liter of water from 15°C. to 16°C.

Heat of Combustion in a Calorimeter.

1 gram of Hydrogen gas produces 34.5 Calories.
1 gram of Charcoal produces 8.0 Calories.
1 gram of Starch produces about 4.2 Calories.
1 gram of Glucose produces about 3.74 Calories.
1 gram of Sucrose produces about 3.96 Calories.

Heat of Combustion in Animals.

1 gram of average Carbohydrate produces about 4.1 Calories.
1 gram of average Fat produces about 9.45 Calories.
1 gram of average Animal Protein produces about 4.25 Calories.
1 gram of average Vegetable Protein produces about 3.98 Calories.
1 gram of average Protein produces about 4.1 Calories.

Oxidation of Carbohydrates.—Carbohydrates, by the processes of digestion, undergo hydrolytic cleavage and are absorbed through the intestinal wall into the blood in the form of glucose. Under normal conditions the blood contains 0.1 per cent or less of this sugar; over 300 grams are stored as glycogen in the body tissues, principally in the muscles and in the liver. Glycogen is readily reconverted to glucose and it serves as a deposit to be drawn upon in an emergency. Glucose is the favorite body fuel, and is used prodigally when the supply is plentiful and thriftily in periods of starvation.

The oxidation of glucose is represented as follows:

	$C_6H_{12}O_6$	+ $6O_2$	= $6CO_2$	+ $6H_2O$
Molecular weights in grams	180	192	264	108
Heat and volume equivalents	673.2 Cal.	134.34 liters	134.34 liters	
Heat and volume equivalents	5.011 Cal. per 1 liter		1 liter	

Carbohydrates contain hydrogen and oxygen in the same proportion as is found in water. When these substances burn, all of the outside oxygen is used to unite with carbon forming a volume of carbon dioxide equal to the volume of oxygen absorbed. The ratio of the volume of carbon dioxide produced to the volume of oxygen absorbed is known as

the *respiratory quotient* (R. Q.). This has a different value for each of the foodstuffs and serves as a means of determining what substances are being burned. From the above equation we see that the respiratory quotient for glucose is $\frac{CO_2}{O_2} = 1.0$ and that 1 liter of oxygen represents a liberation of 5.011 calories.

In animal calorimetry the heat equivalent of 1 liter of oxygen is generally accepted as 5.047 calories when carbohydrates are burned in the body.

Oxidation of Fats.—The fats and oils of our food are mixtures of palmitin, stearin and olein. These substances are of similar chemical composition and when they undergo oxidation they yield about the same amount of heat. After absorption (see Chapter X) fat passes from the blood to the tissues where it is either burned or stored for future use. Fats have a high fuel value and they are continually being used by the body for this purpose. In the absence of carbohydrates, fats supply over 80 per cent of the body heat.

Fat combustion is usually represented by the oxidation of palmitin as follows:

	$2(C_{51}H_{98}O_6)$ +	$145O_2$	= $102CO_2$ +	$98H_2O$
Molecular weight in grams.....	1612	4640	4488	1764
Heat and volume equivalents.....	15233.4 Cal.	3246.6 liters	2283.9 liters	
Heat and volume equivalents.....	4.655 Cal. per 1 liter		0.704 liters	

From the above equation we see that when palmitin is burned the respiratory quotient is 0.704 and that 1 liter of absorbed oxygen liberates 4.655 calories.

Zuntz and Schumburg obtained slightly different figures for the oxidation of fat in the body. In their work the respiratory quotient was found to be 0.707 and the heat value for 1 liter of oxygen 4.686 calories.

Protein Metabolism.—Protein plays a much more important role in general metabolism than the part taken by carbohydrate and fat; the latter are simply stores of body fuel; protein is the fundamental chemical basis of living cells. During life a certain amount of "wear and tear" is continually going on, cell protoplasm is being broken down and must be replaced; internal secretions must be produced for the protection of the organism and for the control of its functions; protein is the substance which the body uses for building purposes.

Although protein is not primarily a fuel, nevertheless when the need arises 58 per cent of its weight can be converted to glucose. Ordinarily we ingest about 100 grams of protein daily. This is probably considerably more than is necessary for the maintenance of health. As is the

case with other foodstuffs the body keeps a store of "deposit protein," which is probably in the form of amino acids. The liver is the chief repository for stored protein.

Proteins in the gastrointestinal tract are completely broken down into the various amino acids and in this form they are absorbed into the blood and are carried to the tissues (see Chapter XIV on Absorption). Amino acids which are not used for the repair of protoplasm or for "deposit" are split into simpler substances of which 58 per cent is oxidized or built into glucose and fat, the remainder containing nitrogen and sulphur is excreted as waste products in the urine and feces.

A computation of protein metabolism by Loewy[1] is as follows:

100 grams of meat protein contain:
52.38 gm. C 7.27 gm. H 22.68 gm. O 16.65 gm. N 1.02 gm. S
of which is eliminated in the urine:
9.406 gm. C 2.663 gm. H 14.099 gm. O 16.28 gm. N 1.02 gm. S
in the feces:
1.471 gm. C 0.212 gm. H 0.889 gm. O 0.37 gm. N
leaving a residuum for the respiratory process of:
41.5 gm. C 4.4 gm. H 7.69 gm. O
deduct intramolecular water:
0.961 gm. H 7.69 gm. O
leaving

41.5 gm. C 3.439 gm. H for oxidation.
When CO_2 is formed 12 gm. C unites with 32 gm. O

Therefore 41.5 gm. C unites with $\frac{32 \times 41.5}{12}$ = 110.67 gm. O

When H_2O is formed 2 gm. H unites with 16 gm. O.

Therefore 3.439 gm. H unites with $\frac{16 \times 3.439}{2}$ = 27.512 gm. O

Total oxygen absorbed..... 138.18 grams.
Total carbon dioxide produced 41.5 gm. C + 110.67 gm. O = 152.17 grams.

In the above computation 1 gram of urinary nitrogen represents:

$$\frac{100 \text{ gm. protein}}{16.28} = 6.15 \text{ grams of muscle protein.}$$

$$\frac{138.18 \text{ gm. } O_2}{16.28} = 8.49 \text{ grams of oxygen absorbed.}$$

$$\frac{152.17 \text{ gm. } CO_2}{16.28} = 9.35 \text{ grams of carbon dioxide produced.}$$

6.15 g. protein × 4.25 (Cal.) = 26.14 calories.
8.49 g. O_2 × 0.6997 (liters) = 5.94 liters of oxygen for protein.
9.35 g. CO_2 × 0.5089 (liters) = 4.76 liters of carbon dioxide from protein.

$$\text{Respiratory quotient for meat protein} = \frac{4.76}{5.94} = 0.801$$

[1] Lusk: Science of Nutrition, p. 39, 1917.

Based on the analytical figures for the average protein it is estimated that 1 gram of urinary nitrogen represents the metabolism of 6.25 grams of protein, the absorption of 5.91 liters of oxygen, the production of 4.76 liters of carbon dioxide, and the liberation of 26.51 calories.[1]

An example of the practical application of the above *constants* is as follows:

A man (aged 38.5 yrs., height 159 cm., weight 90 lbs.) was tested 14 hours after the last meal. An analysis of the urine showed an excretion of 0.16 grams of nitrogen per hour. The total respiratory exchange showed an absorption of 12.52 liters of oxygen and the production of 9.3 liters of carbon dioxide per hour.

0.16 gram of urinary nitrogen represents,

$0.16 \times 6.25 = 1$ gram of protein metabolized with the absorption of

$0.16 \times 5.91 = 0.95$ liter of oxygen, the production of

$0.16 \times 4.76 = 0.76$ liter of carbon dioxide, and the liberation of

$0.16 \times 26.51 = 4.24$ calories

Total CO_2 (or O_2) − protein CO_2 (or O_2) = non-protein CO_2 (or O_2)

9.3 liters CO_2 − 0.76 liter CO_2 = 8.54 liters CO_2

12.52 liters O_2 − 0.95 liter O_2 = 11.57 liters O_2

$$\frac{8.54 \text{ liters } CO_2}{11.57 \text{ liters } O_2} = 0.74 \text{ (non-protein R. Q.)}$$

From Table No. 2 Appendix we find that when the non-protein R.Q. is 0.74, 1 liter of O_2 represents the liberation of 4.727 calories. We also find that 12 per cent of the non-protein heat comes from carbohydrate and 88 per cent from fat.

11.57 liters $O_2 \times 4.727 = 54.69$ calories (non-protein).

12 per cent of 54.69 = 6.56 calories from carbohydrate.

88 per cent of 54.69 = 48.13 calories from fat.

54.69 + 4.24 (protein calories) = Total of 58.93 calories per hour.

In this computation 7 per cent of the body heat was derived from the combustion of protein, 11 per cent from carbohydrate, and 82 per cent from fat.

Respiration.—The ventilation of the lung and the exchange of gases between the alveolar air and the blood are usually referred to as *external respiration*, as contrasted with the exchange of gases in the tissues known as *internal respiration*. Any disproportion between external and internal respiration affects the oxygen or carbon dioxide content of the blood. The tension of oxygen in the blood is lowered when the

[1] Lusk: Science of Nutrition, p. 39, 1917.

lungs are extensively disordered, as in pneumonia, and during periods of supreme muscular effort; under these conditions the oxygen requirements of the tissues are greater than the oxygenating power of the lung. An unusual increase in the supply of oxygen in the lung has but a very slight effect on the tension of oxygen in the blood, and practically no effect on the rate of combustion in the tissues. Of greater interest is the carbon dioxide tension of the blood, which is nicely regulated by an adjustment of the ventilation of the lung, and of the circulation of the blood, so that these mechanisms parallel the rate of combustion in the tissues.[1]

There is a general parallelism between the heat production, the heart rate, and the ventilation rate. It must be remembered that the heart rate is not solely determined by the requirements of respiration, and that ventilation can be voluntarily controlled. A rapid heart and an increased ventilation rate make one suspect that the metabolism is increased, but the rate of heat production cannot be predicted from these phenomena.

External Respiration.—Atmospheric air is of very constant composition the world over. Advantage is taken of this fact in computing the *respiratory exchange*, that is, the amount of oxygen absorbed and the amount of carbon dioxide produced in a given time.

Atmospheric air has the following composition:

Carbon dioxide	0.03 per cent
Oxygen	20.94 per cent
Nitrogen	79.03 per cent

Under ordinary conditions, at each breath a person inspires about 500 c.c. of air and of this amount only 350 c.c. actually enters the lung and diffuses more or less with the 3000 c.c. already there. The expired air includes the 140 c.c. of the "dead space" and 350 c.c. of partially mixed air from the lung (see Fig. 148). Expired air contains about 16 per cent oxygen, 4 per cent carbon dioxide, and 80 per cent nitrogen.

If we exhale forcibly and collect the last portion, the so-called alveolar air, we find that it contains about 14.5 per cent of oxygen and 5.6 per cent of carbon dioxide. That is, the air deep in the lung, where it comes in close proximity to the blood, has the greatest concentration of carbon dioxide and the least concentration of oxygen. These gases are in equilibrium with the gases in the arterial blood.

In Fig. 148 one sees that after an ordinary expiration, 1500 c.c. of additional air can be forcibly expired; then, if the greatest possible inhalation is made, almost 4 liters of fresh air can be drawn into the lungs. This greatly dilutes the carbon dioxide in the alveolar air and

[1] For further discussion of the gas exchange in blood and tissues, see Chapter XIX.

induces a rapid passage of the gas from the blood. If forced breathing is continued, a large amount of carbon dioxide, which is ordinarily stored in the blood, is in this manner "washed out" and may be erroneously regarded as carbon dioxide produced during a period of a metabolism test.

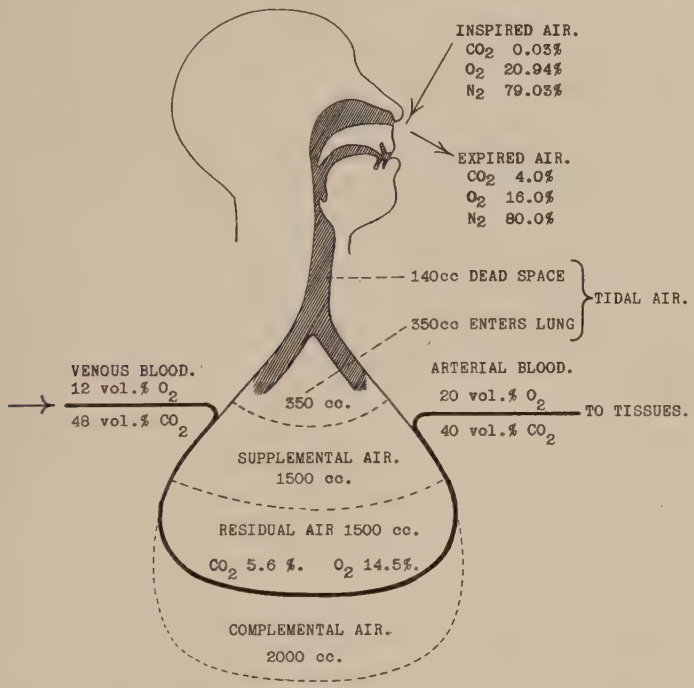


FIG. 148.—INSPIRED AND EXPIRED AIR.

The *tidal air* is that which enters and leaves the body with each normal respiration. The *supplemental air* is that which can be forcibly expired after a normal tidal expiration; this leaves in the lungs the *residual air* which cannot be voluntarily expelled. The *complemental air* is that which can be forcibly drawn into the lungs over and above a normal tidal inspiration. The *vital capacity* is the greatest volume of air which can be expired following a forced inspiration; it includes the tidal, the supplemental, and the complemental volumes.

The *inspired* or atmospheric air is of constant composition. The composition of the *expired air* is not constant; it is determined by the varying factors of combustion in the tissues, the rate of blood flow, and the rate of ventilation in the lung. The *alveolar air* is the last portion of the supplemental air to be expired, and represents the composition of the residual air, and its gases are in equilibrium with the arterial blood. The blood in its passage through the lung gives off carbon dioxide and absorbs oxygen.

The increased supply of oxygen due to forced ventilation has no effect on metabolism tests, in fact, as it has previously been stated, one can breathe pure oxygen without appreciably increasing its absorption. This fact is taken advantage of in the "closed circuit" types of respiration apparatus which require the rebreathing of air, rich in oxygen.

Nitrogen plays a passive role in respiration, the amount expired is the same as the amount inspired. As there is a definite proportion

between the oxygen and the nitrogen of the atmosphere, it is possible to calculate the amount of oxygen inspired on the basis of the nitrogen in the expired air. (See Table 5, Appendix.) The formula is:

$$V = \frac{20.94 \times \text{per cent } N_2 \text{ in expired air}}{79.03}.$$

when V = volume of oxygen in inspired air corresponding to 100 volumes of expired air.

It must be emphasized that in order to measure successfully the carbon dioxide produced during a metabolism test of short duration, *ventilation must be an involuntary act.*

Basal Metabolism

This is an expression of the heat production of the body while completely at rest and in the post-absorptive state, such a condition as is found upon awakening in the morning. For a successful test the subject must be in a "comfortable" environment, if "too hot" or "too cold" the result will be abnormally high. The body temperature must be within the normal limits and the patient must not be one who suffers from daily fevers. DuBois estimates an increase in metabolism of 7.2 per cent for each degree Fahrenheit in fevers. The patient must be peaceful. Mental activity alone has but a slightly elevating effect upon the heat production. Emotional excitation will raise the metabolism 20 per cent or more. This is probably due to the stimulation of the suprarenal glands as suggested by Aub. The emotional factor constitutes the greatest source of error in basal metabolism determinations. Technical errors have been reduced to a negligible quantity. It is not difficult to insure muscular repose and physical comfort, but it requires the utmost sympathy and tact to allay the apprehensions of the patient and insure physiological repose. A common practice is to repeatedly test the patient and to accept the lowest or the last result as the true basal rate; the fallacy of this is obvious when one considers that the number of such tests is usually determined by the endurance of the patient. Tested under the most favorable conditions it is possible, in unstable individuals, to get variations as high as 6 per cent in as many hours. These variations are physiological, not technical; the technic is more accurate than our ability to recognise or control emotional changes.

Clinical Interpretation of Basal Metabolism.—In 1893 the clinical use of calorimetry was presaged by Friedrich Müller who observed that patients with Graves' disease lost weight and had a marked nitrogenous waste despite the fact that the diet was adequate to maintain a normal

state of nutrition. This observation was soon verified by Magnus-Levy who determined the now well known action of the thyroid gland in regulating the rate of combustion in the body. It is true that other factors affect the rate of heat production and that certain well recognized diseases are accompanied by metabolic changes, but *in the majority of cases variations in the basal metabolism can be interpreted as variations in the function of the thyroid gland.*

In attempting to interpret basal metabolism, one cannot do better than recall the statement of Benedict that "basal metabolism is by no means a fixed and definite thing. In spite of the experimental evidence thus far accumulated and the large number of apparatus in operation, our knowledge of basal metabolism is still confined to general trends, and the danger of using these general trends is that clinicians will too readily assume that they represent the conditions to be expected in individual cases."

In an extensive study of basal metabolic rate determinations, Boothby and Sandiford[1] showed that 92.1 per cent of normal individuals have a basal metabolic rate within ±10 per cent, and 99.3 per cent within ±15 per cent of the DuBois standards. They found that a smaller percentage of these same subjects had basal metabolic rates within the same limits when the Harris and Benedict standards were used.

In clinical cases the basal metabolism may vary from 40 per cent below to 130 per cent above the average normal. If no other cause can be found for an abnormal heat production, the result may be cautiously attributed to a disordered activity of the thyroid gland. Glands other than the thyroid may affect the rate of cellular combustion, but at the present time we know of two body substances only which change the rate of heat production, namely, thyroxin and adrenaline. The latter has a rapid, but fleeting, stimulating effect; the effect of the former lasts for several weeks. When the activity of the suprarenal glands is decreased, as in Addison's disease, the basal metabolism is found to be subnormal. A hyperactivity of these glands may account for the temporary increase in metabolism occasionally found in nervous patients, who also show corresponding variations in pulse rate and in blood pressure.

Basal metabolism does not establish a diagnosis of hyperthyroidism. A patient afflicted with hyperthyroidism may be tested during an intermission and the basal metabolism would be within the normal limits despite the persistence of such clinical signs as nervousness, palpitation, tremors and exophthalmos. Conversely, the test may detect active

[1] Boothby and Sandiford: *Jour. Biol. Chem.*, 54, 767 and 783, 1922.

hyperthyroidism in patients showing few of the classical symptoms of the disease. The test does not tell the surgeon when it is safe to operate upon the patient. It informs him when it is the least dangerous to operate and the surgeon must base his operative prognosis upon the clinical condition of the patient and not upon the basal metabolic rate. The debilitated patient whose heart and nervous system have been damaged by repeated thyro-intoxications is a poor risk even though the hyperthyroidism has subsided, whereas the strong, undamaged subject may survive operative procedures at a time when the thyroid is very active.

The onset of hyperthyroidism is accompanied by a progressive increase in heat production which may reach over 100 per cent above the average normal and persist for weeks after the glandular activity has subsided. Therefore, repeated tests are necessary in order to follow the course of the disease; an increasing rate indicates an active state of the gland and naturally precludes operative intervention; a decreasing rate gives a more hopeful outlook.

The pulse rate is largely dependent upon the rate of metabolism. Clinically, a decreasing pulse rate indicates a subsidence of the hyperthyroidism. Few patients with pulse rates below 60 have a basal metabolic rate above the average normal and few patients with pulse rates below 85 have any considerable increase in their metabolism. Beyond that, by a consideration of the pulse rate alone, one can draw no conclusions as to the activity of the thyroid gland. The course of the disease and the result of treatment are accurately represented by changes in the metabolic rate. Cessation of hyperthyroidism is followed by a return of the metabolism to within the normal limits irrespective of the persistence or disappearance of clinical symptoms which may be the more or less fixed signs and symptoms of change due to the prolonged intoxication.

A hyperthyroid patient may have a normal basal metabolism due to the self administration of iodine. An obese patient may have an elevated metabolism from the use of thyroid extract. The basal metabolism probably expresses the toxicity of the thyroid gland, but does not tell us the clinical condition of the patient. In this discussion, a tabulation of the findings in various disorders has been avoided as it is very misleading to the clinician.

Heat comes from oxidation in the active protoplasmic tissue of the body. The rate of heat production is not determined by surface cooling as one method of measurement would seem to imply; in reality, heat dissipation is regulated according to heat production. Any rapid change in the relative amount of protoplasmic tissue will cause changes

in the basal metabolic rate when expressed in terms of weight or surface area. This partly explains the gradual drop in the basal metabolic rate during the glandular and muscular waste of starvation and in hospital patients during their early confinement to bed. A rapid accumulation of fluid in the body has a similar effect and it is quite common to find nephritics with marked edema having a basal metabolic rate of 20 to 30 per cent below the average normal. This must not be interpreted as a sign of hypothyroidism.

Under the basal conditions we measure the heat resulting from activties of the vital organs and from the intracellular chemical changes associated with life, growth and development. These facts must be borne in mind and due allowance made for any unusual activity before the results are attributed entirely to the thyroid gland. Dyspnoea, hypertension, cardiac decompensation, and tremors involve increased muscular activity and so raise the heat production. Certain diseases are characterized by increased cellular activity and in these cases the heat production is increased. The most outstanding of this group is leukemia in which disease the basal metabolic rate may be as high as in severe Graves' disease. Polycythemia, anemia and Paget's disease of the bones are other conditions in which the increased heat production probably results from increased cellular activity. In this group possibly belongs the acute stage of acromegaly. In these conditions the basal metabolism is rarely over 25 per cent above the average normal.

From birth to the age of one and a half years the basal metabolism increases at a remarkable rate. This is followed by a gradual decline until full growth and development is attained; constancy characterizes the rate in adult life, with a slight decline as old age advances. Variations in the rate of growth and development in childhood cause abnormal results when judged by the age standard. In prematurely developed children the basal metabolic rate is low according to the age standard, but is probably normal for the stage of development if this could be accurately expressed. The difficulty in applying the test to children has resulted in a paucity of normal tests and great variation in the results obtained. The normal biological variations are much greater in children than in adults and may be greater than the pathological changes anticipated by the physician. Fortunately the test is rarely required before the age of puberty and the normal standards beyond that age are fairly well established. It must be born in mind that the so called basal metabolism is the measurement under standard conditions of the activity of the vital organs, the tonus of muscle, and the whole influenced more or less by the emotional state of the patient. A person repeatedly tested as a laboratory subject will have a lower

basal metabolic rate than the same individual in the role of a patient. As a clinical test, should we not accept as "standard" the findings in apparently normal patients rather than the findings in apparently normal laboratory subjects? This applies especially to biologically unstable childhood.

The clinical application of the test is greatly restricted, but within its restriction it is one of the most accurate and valuable tests in medicine. The work is highly technical, and unless it is carefully done by experienced persons it will show errors of 20 per cent or more. All laboratory tests should be regarded simply as an aid to the clinical sense of the physician; no diagnosis can be made by machinery alone, and no machine has been devised that will take the place of honest careful analytical work.

Standards of Normal Basal Metabolism and Calculations.—The heat production of normal individuals under basal conditions largely depends upon the factors of age, sex, height, and weight. At the present time, based on these four factors, there are three different systems of predicting the normal heat production.

(1) *Aub and DuBois*[1] determine the heat production in relation to the surface area of the body; the surface area is estimated by the DuBois and DuBois[2] formula which is based on the height and weight: $A = Wt^{0.425} \times Ht^{0.725} \times 71.84$, where A equals the area in square centimeters, Wt, the weight in kilograms, and Ht, the height in centimeters. In routine practice the nomogram of Boothby and Sandiford[3] (Fig. 149) or DuBois' chart (Fig. 150) may be used for determining the surface area.

Stoner[4] has computed tables of values for this formula in unit cm. and kg. intervals for the height range of 110–200 cm. and the weight range of 20–110 kg.

The DuBois smooth line curves and tables show the effect of age and sex upon the heat production per hour per square meter of surface.

These standards have been criticized by many authorities on the ground that the Aub and DuBois table (see Appendix, Table 6) gives the heat production for males and females by decades, instead of making the proper interpolations for intervening years, as represented in the smooth curve from which the data were obtained. The values given by Bailey (Table 3, Appendix) are similar to those of Aub and DuBois and have the advantage of escaping this criticism.

[1] Aub and DuBois: *Arch. Int. Med.*, 19, 823, 1917.
[2] DuBois and DuBois: *Arch. Int. Med.*, 17, 865, 1916.
[3] Boothby and Sandiford: *Boston Med. and Surg. Jour.*, 185, 337, 1921.
[4] Stoner: *Jour. Lab. Clin. Med.*, 11, 355, 1926.

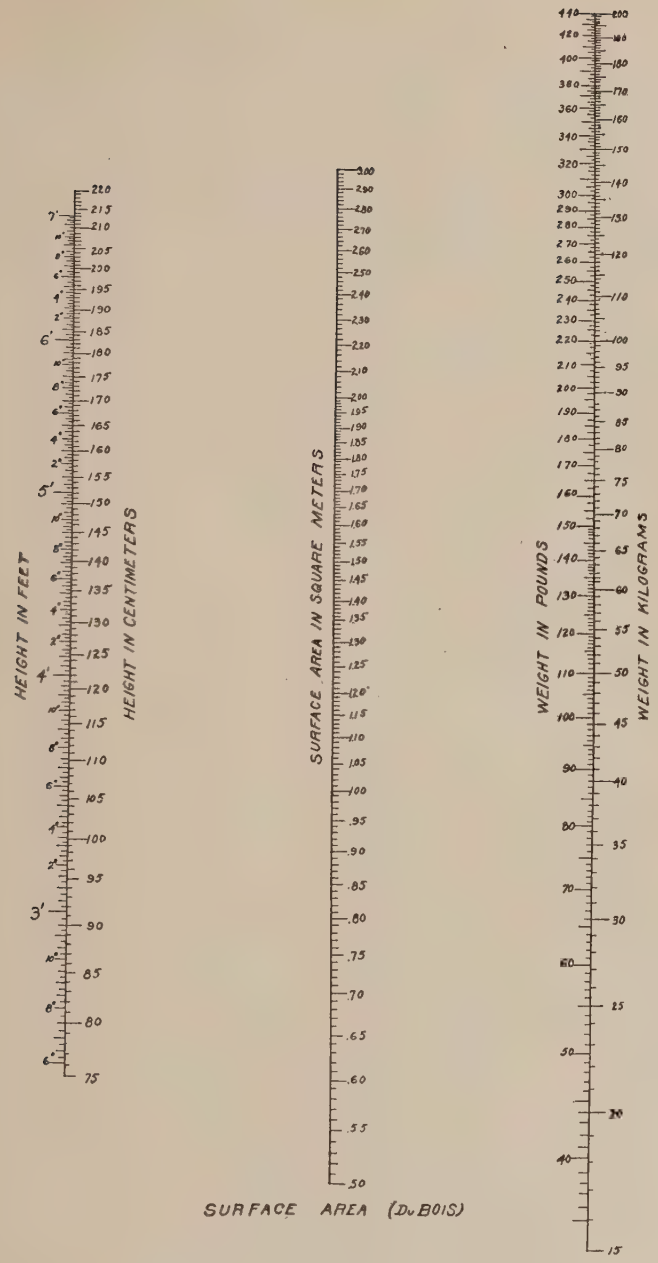


FIG. 149.—SURFACE AREA NOMOGRAM.

(*Copyright* 1920 *by Boothby and Sandiford.*) A line connecting the height with the weight intersects the middle line at the corresponding surface area.

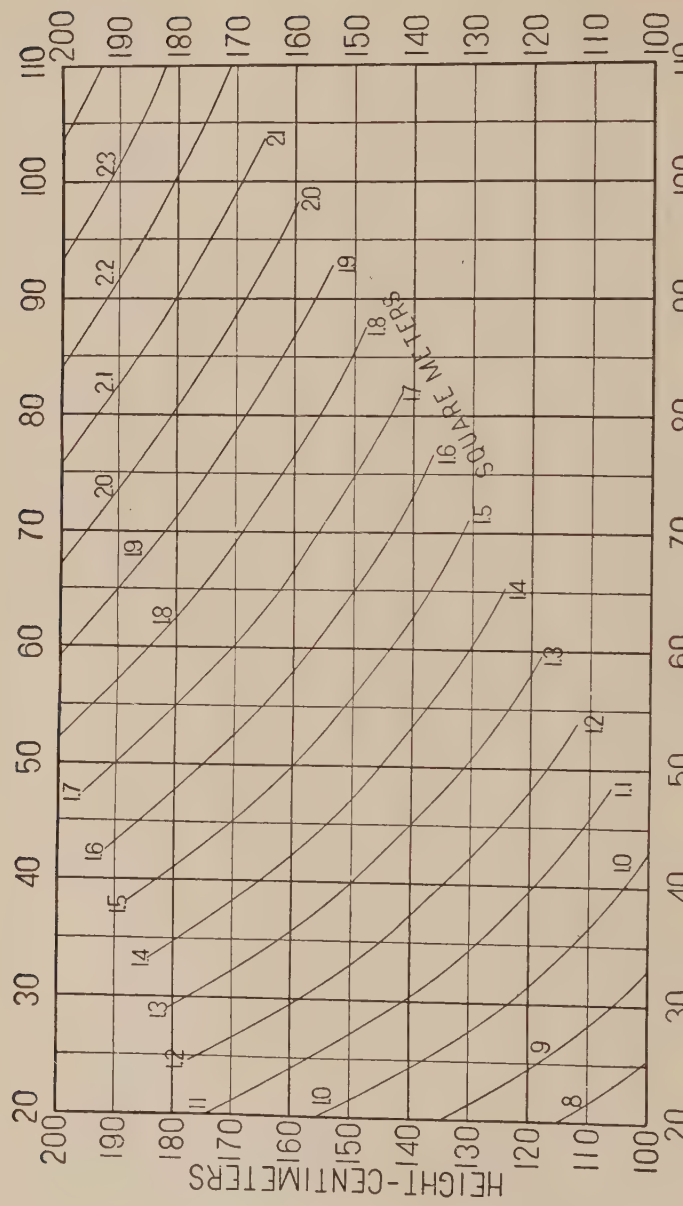


FIG. 150.—DUBOIS SURFACE AREA CHART.

(2) *The Harris-Benedict*[1] multiple prediction equations and tables[2] are based on a statistical study of the available data for the basal metabolism of normal men and women. The equations are,

$$\text{For men, } H = 66.4730 + 13.7516w + 5.0033s - 6.7550a.$$
$$\text{For women, } H = 655.0955 + 9.5634w + 1.8496s - 4.6756a.$$

in which H = total heat production per 24 hours, w = weight in kilograms, s = stature in centimeters, and a = age in years.

(3) From a study of the normal cases reported by Magnus-Levy, by Benedict, and by DuBois, Dreyer[3] devised the following formulas for predicting the normal heat production,

$$\text{For males, } D = \frac{\sqrt{W}}{0.1015A^{0.1333}}$$

$$\text{For females, } D = \frac{\sqrt{W}}{0.1127A^{0.1333}}$$

D = total calories per 24 hours, W = body weight in grams, and A = the age in years.

These methods have been critically studied by Means and Woodwell,[4] Boothby and Sandiford,[5] and by Krogh.[6] The concensus of opinion seems to be that the Harris-Benedict predictions are the most reliable for average normal subjects; that the DuBois standards give results in average normal cases about 4 per cent too high; that in all cases, including those of exceptional build and age, the DuBois standards show less deviation than any of the others.[7]

The method of predicting the normal heat production, and the normal standards, for infants and children are in such an unsettled state that their discussion is beyond the scope of this book. The subject has been reviewed by Talbot.[8]

Table 3[9] shows the results in 1112 apparently normal individuals, tested by the gasometer method. The results are expressed in calories

[1] Harris and Benedict: Carnegie Institution of Washington, Publication No. 279, 1919.

[2] Tables which greatly simplify the use of these formulae may be found in Carpenter, Carnegie Institution of Washington, Pub. No. 303, 1921.

[3] Dreyer: *Lancet*, 2, 290, 1920.

[4] Means and Woodwell: *Arch. Int. Med.*, 27, 608, 1921.

[5] Boothby and Sandiford: *Jour. Biol. Chem.*, 54, 767, 1922.

[6] Boothby and Sandiford: *Physiol. Rev.*, 4, 69, 1924.

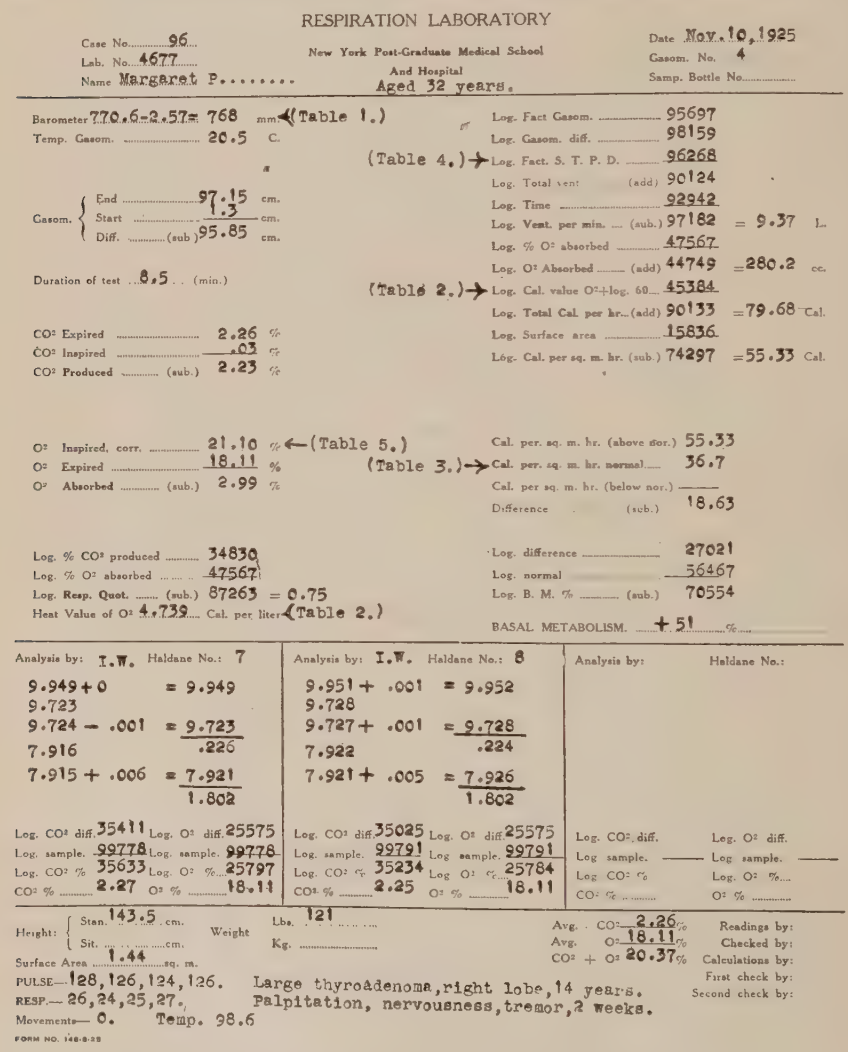
[7] While the DuBois formula indicates a definite mathematical relationship to exist between the surface area and the quantity of energy produced, it has been disputed whether this is a true physiological relation. Benedict, the chief opponent of the surface area law, suggests, however, that "From the practical standpoint it is perhaps not a matter of importance whether the rate of metabolism is directly proportional to the surface area, whether it is controlled by the heat loss from the body, whether the heat loss is independent of the heat supply, or whether the heat produced is determined by the active mass of protoplasmic tissue and the stimulus to the cells. But it is important to know whether there is a referable basis which can be used intelligently for comparing various individuals." Lectures on Nutrition, p. 51, Saunders, Philadelphia, 1925.

[8] Talbot: *Physiol. Rev.*, 4, 477, 1925.

[9] Appendix.

per square meter of surface per hour according to the DuBois formula. The values are practically the same as the normal standards of Aub and DuBois.

Calculations.—In the calculation of the basal metabolism and respiratory quotient five place logarithms are used throughout. The



RESPIRATION LABORATORY

New York Post-Graduate Medical School And Hospital

Case No. 96 | Lab. No. 4677 | Name Margaret P..... | Aged 32 years. | Date Nov. 10, 1925 | Gasom. No. 4 | Samp. Bottle No.

Barometer 770.6-2.57= 768 mm. (Table 1.)
Temp. Gasom. 20.5 C.
Gasom. End 97.15 cm. Start 1.3 cm. Diff. (sub) 95.85 cm.
Duration of test 8.5 (min.)
CO^2 Expired 2.26 %
CO^2 Inspired .03 %
CO^2 Produced (sub.) 2.23 %
O^2 Inspired, corr. 21.10 % (Table 5.)
O^2 Expired 18.11 %
O^2 Absorbed (sub.) 2.99 %
Log. % CO^2 produced 34830
Log. % O^2 absorbed 47567
Log. Resp. Quot. (sub.) 87263 = 0.75
Heat Value of O^2 4.739 Cal. per liter (Table 2.)

(Table 4.)
Log. Fact Gasom. 95697
Log. Gasom. diff. 98159
Log. Fact. S. T. P. D. 96268
Log. Total vent (add) 90124
Log. Time 92942
Log. Vent. per min. (sub.) 97182 = 9.37 L.
Log. % O^2 absorbed 47567
Log. O^2 Absorbed (add) 44749 = 280.2 cc.
(Table 2.)
Log. Cal. value O^2+log. 60 45384
Log. Total Cal. per hr. (add) 90133 = 79.68 Cal.
Log. Surface area 15836
Log. Cal. per sq. m. hr. (sub.) 74297 = 55.33 Cal.

Cal. per. sq. m. hr. (above nor.) 55.33
(Table 3.) Cal. per. sq. m. hr. normal 36.7
Cal. per sq. m. hr. (below nor.)
Difference (sub.) 18.63

Log. difference 27021
Log. normal 56467
Log. B. M. % (sub.) 70554
BASAL METABOLISM. + 51 %

Analysis by: I.W. Haldane No.: 7	Analysis by: I.W. Haldane No.: 8	Analysis by: Haldane No.:
9.949 + 0 = 9.949	9.951 + .001 = 9.952	
9.723	9.728	
9.724 − .001 = 9.723	9.727 + .001 = 9.728	
7.916 .226	7.922 .224	
7.915 + .006 = 7.921 1.802	7.921 + .005 = 7.926 1.802	
Log. CO^2 diff. 35411 Log. O^2 diff. 25575	Log. CO^2 diff. 35025 Log. O^2 diff. 25575	Log. CO^2 diff. Log. O^2 diff.
Log. sample. 99778 Log. sample. 99778	Log. sample. 99791 Log. sample. 99791	Log. sample. Log. sample.
Log. CO^2 % 35633 Log. O^2 % 25797	Log. CO^2 % 35234 Log. O^2 % 25784	Log. CO^2 % Log. O^2 %
CO^2 % 2.27 O^2 % 18.11	CO^2 % 2.25 O^2 % 18.11	CO^2 % O^2 %

Height: Stan. 143.5 cm. Sit. cm. Weight Lbs. 121 Kg.
Surface Area 1.44 sq. m.
PULSE 128, 126, 124, 126.
RESP. 26, 24, 25, 27.
Movements 0. Temp. 98.6
Large thyroadenoma, right lobe, 14 years. Palpitation, nervousness, tremor, 2 weeks.
Avg. CO^2 2.26% Avg. O^2 18.11% CO^2 + O^2 20.37%
Readings by: Checked by: Calculations by: First check by: Second check by:
FORM NO. 146-8-25

FIG. 151.—FORM SHEET FOR STUDY OF RESPIRATORY METABOLISM.

characteristics, or the figures before the decimal points of the logarithms which fix the decimal points of the numbers, are omitted. The decimal point in the final result is determined by inspection of the numbers employed. For example, the carbon dioxide produced is 2.23, the oxygen absorbed is 2.99; the respiratory quotient ($CO_2 \div O_2$) must be

somewhat less than 1; it is 0.75. The sources of error in routine calculations are greatly reduced by the use of the form sheet devised by Boothby and Sandiford.[1] An example of such a calculation is given in Figure 151. Reference is made to the various tables which are used to simplify the procedures (see Appendix). Results are usually reported as the Basal Metabolism or Basal Metabolic Rate[2] (B.M.R.) which may be defined as the percentage variation of the *observed*, above or below the *predicted* basal heat production for individuals of given height, weight, age and sex.

The calculation for basal metabolic rate may be simply expressed by the following formulas[3] which are useful even in the absence of a kymograph.

1. Using the normal standards of Aub and DuBois:[4]

$$B.M.R. = \frac{10.4pv}{mtSA} - 100$$

in which p is the corrected barometric pressure in mm. of mercury, v the actual observed[5] volume of oxygen consumed in the basal period, in liters, m the time of the period in minutes and fractions thereof, t the temperature in degrees centigrade, S the surface area in square meters and A the heat production per hour per square meter according to the Aub and DuBois standards. 10.4 combines the constants in the equation, and corrects the volume to normal temperature and pressure, converts from a minute to an hour basis, converts the oxygen consumption to calories assuming the respiratory quotient to be 0.82, and finally converts to a per cent basis.

2. Using the normal standards of Harris and Benedict:[6]

$$B.M.R = \frac{250pv}{mtH} - 100$$

in which p, v, m, and t have the same significance as above, and H represents the 24 hour heat production according to the normal standards of Harris and Benedict. The constant 250 corrects the volume to normal

[1] Boothby and Sandiford: Basal Metabolic Rate Determinations, W. B. Saunders Co., 1920.

[2] This term, adopted by Boothby, has won popular favor. Du Bois objects to it because it adds an unnecessary word.

[3] Stoner: *Bost. Med. and Surg. Jour.*, 189, 195, 1923. A simplified data card is also described for the use of these formulae.

[4] Aub and DuBois: *Arch. Int. Med.*, 19, 823, 1917. The values given in Tables, 3 or 6, Appendix, may be used in this formula.

[5] Emphasis is laid on the fact that v represents the *actual* volume and not the reading on a special scale, such as is supplied with certain instruments on the market, with the object of correcting the volume read at 20° to its value at 0°C. For such apparatus (Sanborn "Handy Model") the coefficients must be changed to 11.3 (Aub and DuBois) and 268 (Harris and Benedict), respectively.

[6] Harris and Benedict, Carnegie Institution of Washington, Pub. No. 279, 1919 Also in Carpenter: *ibid.*, Pub. No. 303, 1921.

temperature and pressure, converts from a minute to a 24 hour basis, converts the volume of oxygen consumed to calories, assuming the respiratory quotient to be 0.82, and finally converts to a per cent basis.

3. Using the normal standards of Dreyer:[1]

The formula is identical with the Harris and Bendict formula, except that the normal standard of Dreyer (*D*) is substituted for *H*.

Animal Calorimetry.—The rate of combustion may be determined by directly measuring the heat given off by the body. Every animal has an optimum temperature and this temperature is maintained by a nice balance between the heat produced by the life processes and the heat lost by the organism. Gephart and DuBois[2] estimate that in man, 24 per cent of the total heat loss results from the evaporation of water from the lungs and skin. A small amount of heat is lost in warming the ingested food, the urine, the feces, and at times the body itself; the remainder is lost by conduction and by radiation.

Lavoisier and Laplace measured the heat loss by placing a guinea pig in an ice chamber for ten hours and observing the amount of ice melted. During the past thirty years, the elaboration of this simple form of calorimeter has culminated in the construction of the Atwater-Rosa-Benedict Respiration Calorimeter.[3] With this apparatus, the heat of the body is determined indirectly from the respiratory exchange, and directly by a careful computation based on observations of all the known means of heat loss.

The principle of this apparatus was applied by Armsby[4] in the elaboration of a calorimeter for use with farm animals. Respiration calorimeters[5] have provided the means of acquiring most of the present day knowledge of energy metabolism. They have been extremely valuable in proving the accuracy of indirect calorimetry.

Dr. F. G. Benedict of the Nutrition Laboratory of the Carnegie Institution, has been the leader in the development of closed circuit respiration apparatus suitable for use in medical schools and in the clinic. The various types of respiration apparatus introduced in the past ten years are simplifications of his clinical respiration chamber illustrated in Figure 152.

Basal metabolism is usually determined in 10 to 15 minute periods using either one of the Benedict "closed circuit" methods as developed

[1] Dreyer: *Lancet*, 199, 280, 1920. Tables simplifying the use of Dreyer's formula are given by Stoner: *Bost. Med. and Surg. Jour.*, 189, 239, 1923; 191, 1026, 1030, 1924.

[2] Gephart and DuBois: *Arch. Int. Med.*, 17, 902, 1916.

[3] Atwater and Rosa: *Report of Storr's Agric. Exp. Sta.*, 1897, p. 212.
Atwater and Benedict: Carnegie Inst. Wash. Pub. No. 42, 1905.

[4] Armsby and Fries: Bull. 51, U. S. Dept. of Agriculture (Bureau of Animal Industry), 1903.

[5] An historical and descriptive review of respiration calorimeters may be found in DuBois: Basal Metabolism in Health and Disease, Lea and Febiger, 1924.

in the Carnegie Nutrition Laboratory, or a modified Tissot or "open circuit" method as used in the Mayo Clinic and in the New York Post Graduate Hospital. Obviously, the determination of urinary nitrogen in such tests is impracticable. It has been the custom in these institutions to apply the calorific values in Table 2 (Appendix) directly to the

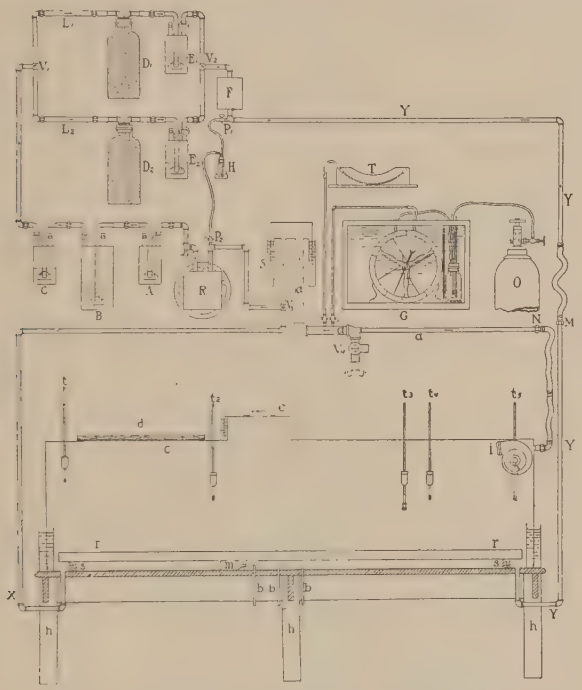


FIG. 152.—DIAGRAM OF THE COT CHAMBER OF DR. F. G. BENEDICT.

The upper part of the figure shows the universal respiration apparatus with the following parts: R, blower; A, acid trap; B and C, water absorbers; V_1 and V_2, two-way valves connecting with the carbon dioxide absorbing system; D_1 and D_2, carbon-dioxide absorbers: L_1 and L_2, removal sections of the piping for the introduction of additional carbon-dioxide absorbers; E_1 and E_2, water absorbers; F, sodium bicarbonate can; P_1 and P_2 and H, petcocks and barium hydroxide container for testing the efficiency of the carbon-dioxide absorbers; Y, Y, tube through which the air freed from carbon dioxide and water returns to the chamber; M and N, points at which the tubing is connected when the apparatus is used with a mouth piece or nose pieces; S, spirometer; G, gas meter immersed in water; O, oxygen tank; T, manometer; V_3 and V_4, three-way valves; a, a, a, tube connecting chamber with spirometer and oxygen supply. The lower part of the figure shows the respiration chamber. X, outcoming air pipe; i, blower; c, window with water-seal d; e, hand-hole; t_3 and t_4, psychrometer thermometers; t_1, t_2, t_3, t_5, air thermometers; h, h, h, supports for chamber; b, b, b, tubes in bottom of chamber for various connections; r, r, bed; s, s, springs; m, pneumatic bulb.

respiratory exchange as measured, without computing separately the protein metabolized. DuBois[1] points out that this procedure gives results about 1 per cent too high.

The *Universal* respiration apparatus dispenses with the chamber and requires the patient to breathe through a mouth piece at V_4 (Fig. 151).

[1] DuBois: Basal Metabolism in Health and Disease, Lea and Febiger, 1924.

The *Benedict Portable*[1] includes all the principal parts of the universal apparatus, in compact form. The gas meter is dispensed with. Oxy-

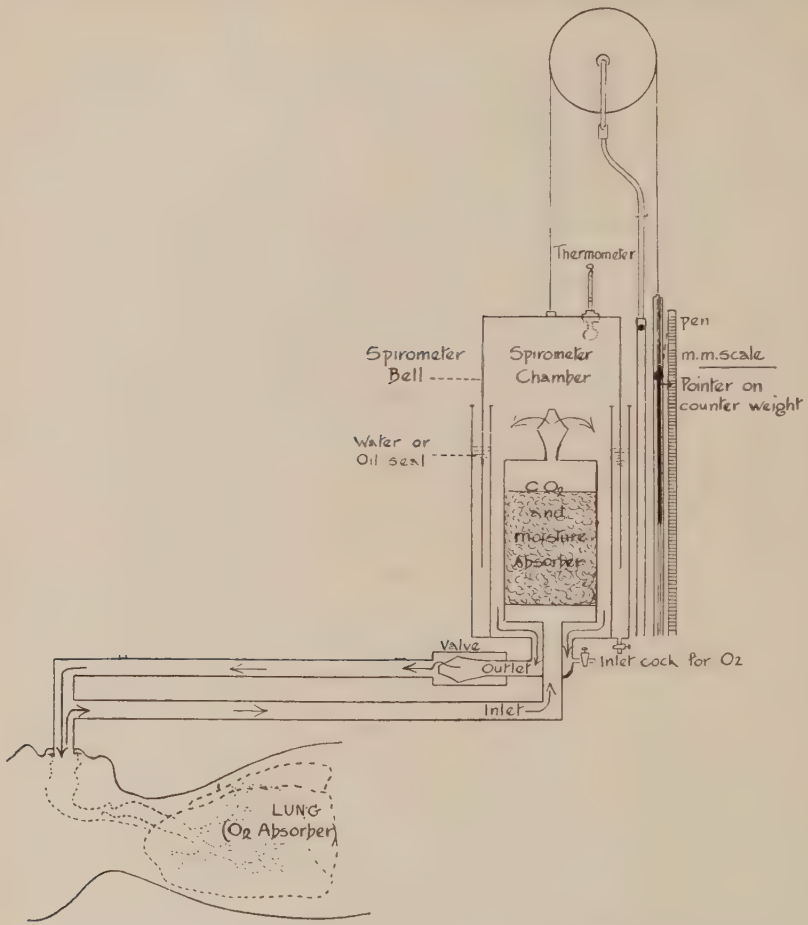


FIG. 153.—DIAGRAM OF THE BENEDICT-ROTH RESPIRATION APPARATUS.[2]

The patient breathes through a mouthpiece. The nose is clamped. Two tubes connect the mouthpiece with the spirometer. An inspiratory Sadd valve directs the oxygen from the spirometer chamber to the patient; an expiratory valve insures its continuous circulation. The expirations pass through soda lime which removes carbon dioxide. The calibrated spirometer bell is balanced by a counterweight which carries a pointer. The pointer shows the movements of the bell on a millimeter scale. A test is started with the chamber full of oxygen; as oxygen is absorbed by the patient the volume in the chamber decreases. The loss of volume represents oxygen absorbed during the period of the test. Correction is made for any change in chamber temperature during the test.

gen is placed in the apparatus before the test is started, and its absorption is measured by a calibrated gasometer. Carbon dioxide is determined by weighing the absorber before and after the test.

[1] Benedict: *Boston Med. and Surg. Jour.*, 178, 667, 1918.
[2] Slightly modified from Roth: *Boston Med. and Surg. Jour.* 186, 491, 1922.

The *Benedict-Collins*[1] is a further simplification of the "Portable." Oxygen absorption only is determined, the respiratory quotient is assumed to be 0.82, and the heat value per liter of oxygen is taken as 4.825 calories.

Benedict-Roth Respiration Apparatus.—(Fig. 153). This form of apparatus is now extensively used in clinics for determining the basal

FIG. 154.—BENEDICT-ROTH RESPIRATION APPARATUS WITH KYMOGRAPH.

metabolic rate of patients. Roth[2] has dispensed with the electric blower of the Benedict machines, and has inserted two Sadd valves to insure circulation of the oxygen. The spirometer bell is cleverly designed to have a volume of 20.73 c.c. for every millimeter of height. This greatly simplifies the calculations. The fall of the spirometer bell during a test of six minutes represents the volume of oxygen absorbed by the patient. As with other Benedict apparatus, the respiratory quotient is assumed to be 0.82; this gives a heat value for 1 liter of oxygen of 4.825 calories. "With a bell of this size, *each mm. of*

[1] Benedict and Collins: *Bos.on Med. and Surg. Jour.*, 183, 449, 1920.
[2] Roth: *Bost. Med. and Surg. Jour.*, 186, 457, 491, 1922.

the fall of the bell in a six minute period represents exactly 1 *calorie per hour.* (1 mm. in 6 min. = 20.73 c.c. = 0.2073 liter per hour. 0.2073 × 4.825 = 1 calorie per hour.)"

The latest form of this apparatus (Fig. 154) is provided with a kymograph which is self time-marking in minute periods. A pen attached to the counterweight traces the respiratory excursions and shows the lowering of the spirometer bell due to the absorption of oxygen. Figure 155 illustrates the method of drawing the "oxygen con-

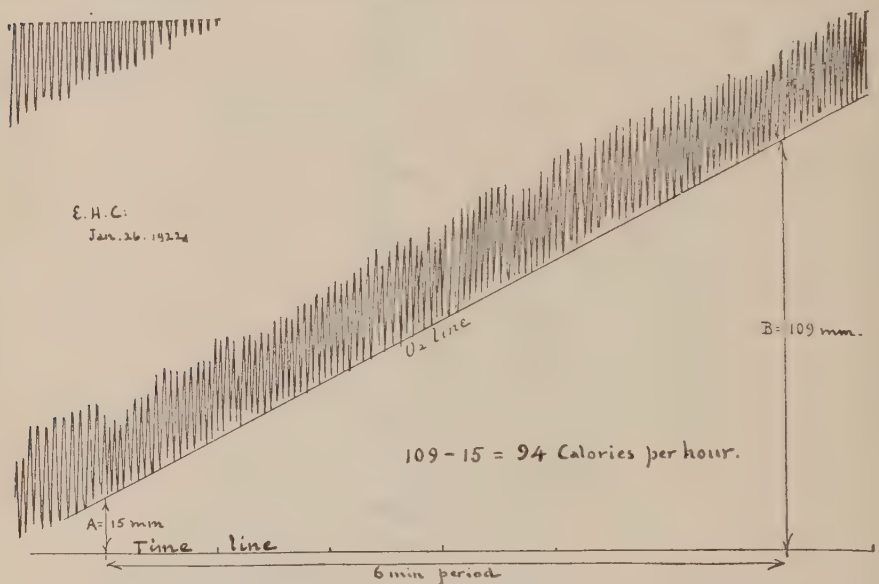


FIG. 155.[1]—Graphic Record of a Metabolism Test. Mr. E. H. C. Aged 38. Height 176 cm. Weight 84 kg. Surface area 2.0 sq. m. Barometric pressure 745 mm. Average temperature 24°C. (no increase during test). Rise of O_2 line in 6 minutes, 94 mm. = 94 calories per hour. Correction for temperature, pressure, and water vapor[2] 94 × 0.879 = 82.63 calories per hour. Divide by surface area 82.63 ÷ 2 = 41.3 calories per square meter of surface per hour, which is 5 per cent above the average normal (39.5) for this individual. (Roth.)

sumption line," and of measuring the "rise" of this line for any 6 minute period which may be selected on the tracing. If the moisture content of the circulating air is high, as is the case when Wilson Soda Lime is used as an absorber, a correction must be made for water vapor. Roth[2] gives the factors for reducing the volume of absorbed oxygen to 0°C., 760 mm., dry (when the vapor tension is 80 per cent of saturation).

[1] A simplified data card for taking record of instruments not equipped with kymographs and for making the calculations, has been described by Stoner, *Bost. Med and Surg. Jour.*, 189, 195, 1923.

[2] A table for reducing volumes of 80 per cent saturated air to 0°C. and 760 mm., dry, is given by Roth, *Bost. Med. Surg. Jour.*, 186, 457, 1922.

Procedure.—The patient is tested before breakfast and after a 12 to 15 hour fast. A record is made of the sex, age, height, and weight. A rest in a semi-reclining position for half an hour must preceed the test. During the rest period, the pulse rate, the respiration rate and the temperature are observed, and the patient is briefly instructed in order to secure his confidence and cooperation. The apparatus is tested for leaks as follows; a stopper is placed in the opening of the mouthpiece, a 200 gram weight is placed on the bell. The pointer should remain stationary for a minute or more. The weight and stopper are removed and the spirometer is filled with oxygen. The mouthpiece and later the nose clip are applied to the patient, care being taken to avoid leaks. The kymograph[1] is started. The temperature of the spirometer, and the barometric pressure are recorded. The test is continued until a satisfactory uninterrupted section of exactly six minutes can be selected for subsequent measurement. Before stopping the test, a weight of 50 grams is placed on the bell and operation is continued for a few minutes; if a leak occurs it is shown by a sharp rise in the oxygen consumption line. The temperature of the spirometer is again recorded. Figure 155 shows a graphic record of a test, and the legend gives the necessary calculations for determining the basal metabolic rate.

Benedict Student Apparatus.[2] (Fig. 156).—*Principle.*—The subject breathes through a mouthpiece connected with two Sadd valves attached to a metal can, three-fourths filled with soda-lime.[3] A rubber bathing cap over the top encloses oxygen-rich air and permits free breathing. The amount of oxygen absorbed from the enclosed volume of air is measured by introducing dry room air into the can with a good grade of automobile grease plunger pump. The time required to introduce six strokes of the plunger is recorded. From the apparent volume, reduced to 0°C. and 760 mm., and the time, the oxygen consumption is rapidly obtained." This apparatus has the advantage of being stripped of blowers, kymographs, diagrams and charts which may mask the simple principles involved, and which may give a clinician a sense of false security.

[1] In the case of spirometers not equipped with kymographs, readings on the scale are made at the beginning and end of the periods. Stoner (*Bost. Med. and Surg. Jour.*, 189, 193, 1923) divides the ten minute period into five two minute periods, taking the average of ten spirometer readings at the beginning of each period. In this way he is able to select a four minute period showing the least oxygen consumption, and also to avoid the error due to wide fluctuations in individual respirations.

[2] Benedict and Benedict: *Boston Med. and Surg. Jour.*, 188, 567, 1923. This apparatus is obtainable from Warren E. Collins, 584 Huntington Ave., Boston, Mass.

[3] Soda Lime. "750 grams of unslaked lime and 750 grams of crude commercial sodium hydroxide are each weighed separately. The caustic soda is dissolved in 450 c.c. of water in an iron pot over a gas flame. When this is thoroughly dissolved the lime, which has been previously pulverized, is carefully but rapidly sifted into the pot, the flame turned down, and the whole mass thoroughly stirred *with a long handled iron rod* until slaking is completed. The finished material should be lumpy and slightly moist to the touch, but not so moist as to be sticky nor so dry as to be powdered easily. After cooling, the material is broken into sizes suitable for use. We have found for this purpose that material which passes through a sieve with a mesh of 4 mm. is best." Benedict and Benedict: *Bost. Med. and Surg. Jour.*, 188, 567, 1923.

Procedure.—Benedict describes the use of the apparatus as follows:

After placing the moistener in the metal pipe connecting the two rubber tubes, the mouthpiece is inserted. The bag is then filled with oxygen through the petcock at the bottom, starting with the bag flat or slightly depressed into the can. This insures enough oxygen enrichment to preclude any possibility of "oxygen want."

Attach the noseclip, preferably at the end of a normal expiration. When the operator does this, it is helpful to watch the rise and fall of the chest of the

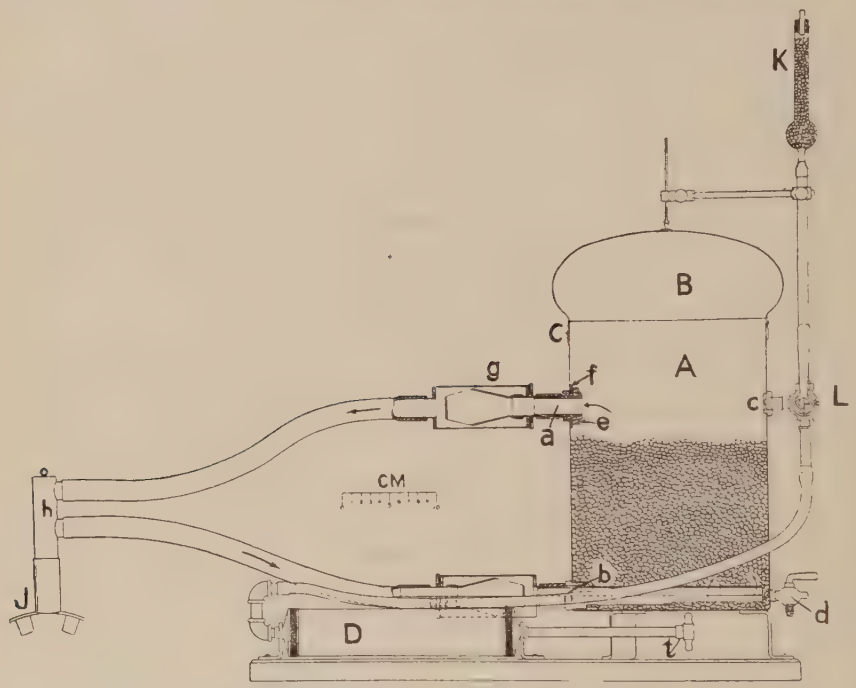


Fig. 156.—Benedict's Student Model Respiration Apparatus.

A, metal can partly filled with soda-lime; *B*, rubber bathing cap, held in place by rubber band, *C*. The subject breathes through the mouthpiece *j*, which is attached to the metallic piece, *h*, connecting with two rubber tubes leading to Sadd respiratory valves on the can, *A*. The expired air enters through the opening, *b*, and is returned to the subject, freed of carbon dioxide, through the opening, *a*. The Sadd valves are enclosed in light brass housings, *g*. The opening, *a*, is stiffened by a lock nut, *e*, and a small, flat piece of iron, *f*, curved to fit the can. Oxygen for preliminary enrichment is admitted through the petcock, *d*, and measured amounts of dry air, drawn through the calcium-chloride tube, *K*, into the pump, *D*, are forced into the can through the valve, *L*, and the opening, *c*.

subject. The subject can, however, as is frequently the case, apply the noseclip himself.

The bag will now rise and fall with each respiration. Enough air should then be introduced by the air pump[1] to fill the bag until it touches the needle, indeed until it bulges slightly around the top. Great excess is undesirable, as it is liable to produce pressure against which the subject must expire. In

[1] Internal diameter about 50 mm., length of stroke about 200 mm., *i.e.* total apparent complete stroke about 400 c.c. Avoid warming the air by placing the hand upon the pump barrel.

using the air pump the valve, L, should be turned so as to connect the pump first with the calcium-chloride tube, K, and the pump handle drawn completely out. The valve should then be turned to connect with the can, and the handle slowly pushed to drive dry air into the can.

At the end of each expiration the button will press less and less against the needle and shortly it will just touch. At this point the stop-watch should be started or the exact time noted on an ordinary watch, recording first the position of the second hand, then the minute and hour.

Fill the air pump with dry air and slowly drive it into the can.[1] Make any major movements of the plunger when the bag is high, i.e., towards the end of expiration, to avoid introducing an excessive amount of air into the bag.

As the plunger nears the end of a complete stroke, drive all the air out of the barrel so that a slight excess is present in the rubber bag with slight indications of distention, and wait, as at the start (see *e* above), until the index just leaves the needle. Note the time but do not stop stop-watch. Record the time required for the introduction of one complete pump stroke in minutes and seconds.

Repeat the above for 6 full strokes, noting the total elapsed time in each case.

At the end of the sixth stroke, after noting the time, remove noseclip and mouthpiece, record the temperature of the pump and the barometer. The temperature of the pump is obtained from an ordinary laboratory thermometer with its bulb touching the brass of the barrel.

Calculation.—The apparent volume of oxygen absorbed during the complete period when six full strokes of the air pump were introduced is computed readily. From the internal diameter of the barrel, the length and number of strokes, the apparent volume of air (K) introduced is computed. This volume is constant for each pump. There is no tension of aqueous vapor to be considered, for the air drawn into the barrel is dried by calcium chloride, and the air over the soda-lime is essentially dry. Thus the apparent volume may be reduced to 0°C. and 760 mm. by the formula

$$V \times \frac{273}{273 + t} \times \frac{p}{760}$$

in which t is the temperature of the pump barrel and p the observed barometer.

Finally, if the reduced volume be divided by the time in minutes,[2] the oxygen consumption per minute (V) is obtained. The calculation[3] thus is more simply expressed as

$$V = \frac{K \times m}{T}$$

in which K is the constant apparent volume of six full pump strokes, m is the reduction factor at t and p, and T the time in minutes. For further details, consult the original paper.[4]

[1] The turning of the 3-way valve may seem confusing, but no error that cannot be immediately rectified can be made if the valve is *turned* only when the pump handle is clear in or clear out. If air is inadvertently drawn out of the bag by pulling the handle out, it may immediately be quantitatively returned to the bag.

[2] It is necessary to convert the time as recorded in minutes and seconds to decimal parts of a minute. Thus, 11 minutes and 27 seconds equal 11.45 minutes.

[3] Standard tables giving the combined factor (m) for reducing volumes of dry air to 0°C. and 760 mm. have been prepared by Roth, and in more detailed form by Carpenter.[5]

[4] Footnote 2, p. 515.

[5] Carpenter: Carnegie Inst. of Wash. Pub. No. 303, 1921.

Example.—A typical experiment was made on a normal subject, a woman, 24 years of age, weighing (nude) 57.2 kilograms, and with a height of 157 centimeters. The room temperature was 19.6°C., the temperature of the pump was 18.3°C., and the barometer stood at 755.9 mm. The elapsed time from the beginning of the experiment to the complete consumption of the oxygen represented by the air discharged at each stroke of the pump, was as follows:

Pump stroke No.	Elapsed time mins.	secs.
1	1	56
2	3	54
3	5	50
4	7	46
5	9	36
6	11	27

Six full strokes with a length of stroke used in this particular apparatus corresponded to 2168 c.c. apparent volume. From the tables for the reduction of volumes of air to 0°C. and 760 mm. it can be seen that at the temperature of the pump, namely, 18.3°C., and with the barometer at 756 mm., the factor is .932. The time for the six strokes is expressed as minutes and decimal parts of a minute, namely, 11.45 minutes. The formula $\frac{K \times m}{T}$ thus equals

$$\frac{2168 \times .932}{11.45} = 176 \text{ c.c. } O_2 \text{ consumed per minute.}$$

Gasometer Method (*As Applied in the New York Post Graduate Hospital*).—Basal metabolism is a measurement of the rate of vital activities under the standard conditions of *emotional* as well as of physical repose. This must be the dominant thought of the examiner and it must not be supplanted by worries over technical details. For an occasional test, any of the existing methods give satisfactory results, provided that the examiner thoroughly understands what he is doing.

In institutions where many determinations are made each day, the gasometer method is the most satisfactory, in that it permits of a division of labor. The clinician can devote his entire attention to the patients, the technical details can be taken care of by assistants in an adjoining room. The method essentially consists of having the patient breathe atmospheric air, the expired air is collected and measured in a gasometer; samples of the collected air are analyzed for carbon dioxide, oxygen, and nitrogen; from these factors the respiratory exchange is calculated. The test permits of a division into three distinct steps, which will be described in detail.

Procedure.—The patients are instructed to eat their ordinary meals on the preceeding day, to avoid a late supper, and to come to the office having partaken of nothing but a glass of water on the morning of the test. Upon arrival, the patient is assured that the test is extremely simple and by no means unpleasant.

Excessive and tight clothing are removed, the height and nude weight are ascertained; the patient is placed on a couch and covered with a sheet and enough blankets to insure comfort.

Collection of Expired Air in Gasometer.[1]—While the subject is resting, the gasometer is emptied until the pointer stands at zero. The air-tube (G) is

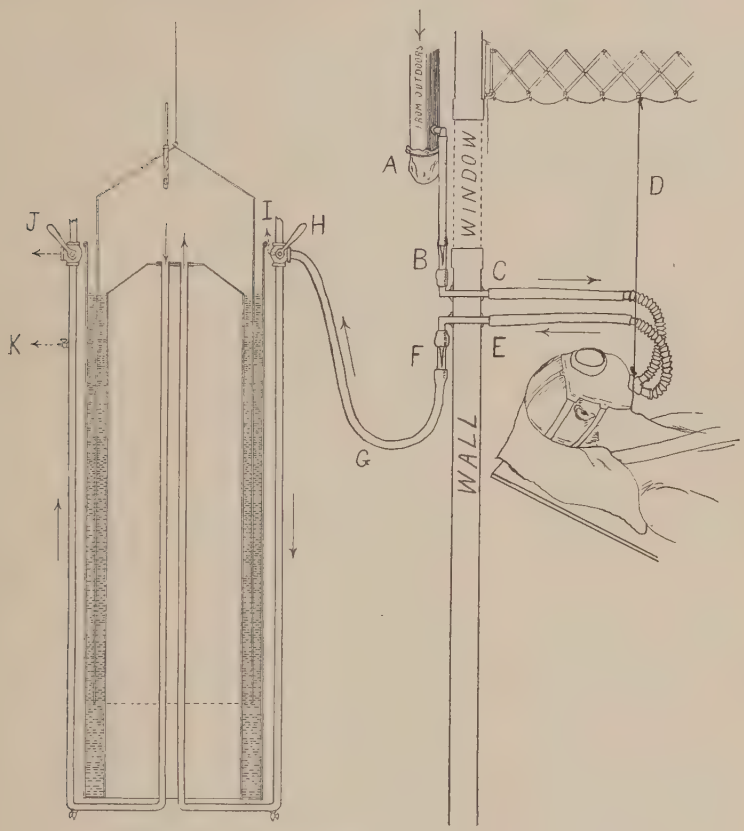


Fig. 157.—Diagram of Gasometer for Collection of Expired Air.

connected with the gasometer, and the rubber cap (A) removed to admit air to intake pipe; the cap is then replaced.

When the patient is sufficiently rested, the tubes attached to the mask are (Fig. 159) connected to the inspiratory and expiratory pipes. The mask is then applied. When accurately fitted, the mask-cord (D) is tied to the overhead hook, holding the mask in the most comfortable position. From the time the

[1] *Pipes, Valves, and Connections.*—Outdoor air enters laboratory through a 4-inch galvanized iron pipe. This pipe is covered with a rubber bathing cap (*A*, Fig. 157) held in place by a rubber band, to prevent wind from blowing through the valves. A length of 24 mm. rubber tubing leads from the large pipe to the inspiratory Sadd valve (see Fig. 157) The Sadd valves are enclosed in flattened glass cases which are mounted directly on the laboratory ends on the wall tubes (*B* and *F*); the latter have elbows directed upward on the inspiratory and downward on the expiratory—this permits both the flutter-valves to hang downward, in which position they functionate most favorably. From the lower end of the expiratory valve-case, a 24 mm. rubber tube (*G*) leads to the gasometer.

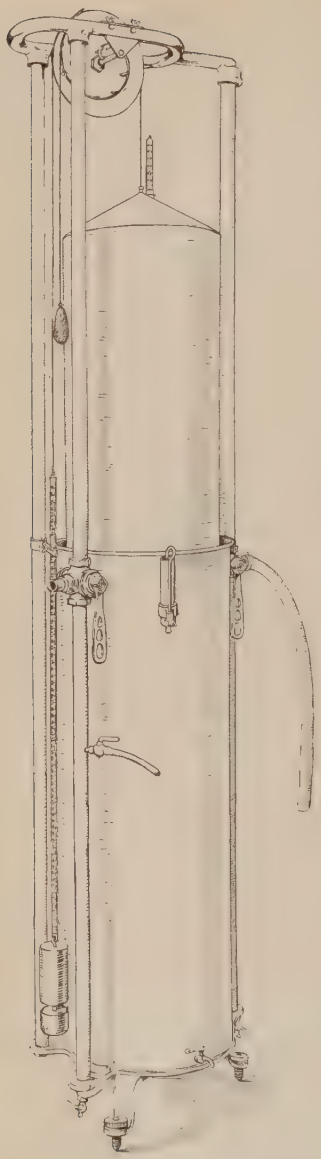


FIG. 158.—BAILEY GASOMETER.

mask is applied, the inspiratory air is drawn from outdoors through the valve (B), and the expiratory air escapes into the room through valve (J). In this way the entire dead space of the apparatus is flushed out with expired air.

One is now ready to start the test. The respiratory phases can be readily followed by observing the valves (B and F). During the inspiratory phase, the valve (H) is quickly turned to communicate with the gasometer and at the same time one presses the stop watch and the collection is begun. While the air is being col-

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The *gasometer*[1] (Fig. 158) is a stationary piece of apparatus, about 8 ft. high and has a capacity of 90 to 100 liters. The copper bell is 100 cm. in length and has a diameter of 34.59 cm. Measuring the rise of the bell to the nearest half-millimeter, a change in volume of 47 c.c. can be detected. The framework is rigidly constructed of iron pipes and castings. At the top it supports the grooved counterbalance wheel, on its flat base rests the copper water-bath. The base contains inlet and outlet passages for air; it is fitted with three levelling legs and threads to receive the supporting and air pipes of the framework. The counterbalance wheel carries a grooved spiral (see below) from which is suspended a compensating weight; as the bell rises from the water and becomes progressively heavier, the leverage of this weight is correspondingly increased, and in this way the bell is exactly counterbalanced in all positions.

The bell is suspended by a piano wire which passes in the groove of the wheel and is continuous with a 100 cm. length of steel measuring tape, to the lower end of which the main counterweight, which is in two sections, is hooked. The tape, which reads in millimeters from below upward, passes through a suitable guide bearing a pointer at which the readings are made. The water-bath is circular in plan and has outer and inner walls, 4 cm. apart; the outer wall is 100 cm. high; the inner wall has a height of 100 cm. and with a truncated conical roof forms the obturator of the bell. In use the bath is kept filled with water to a level of 100 cm. The outlet pipe has a sampling cock placed about a foot below the three-way valve.

Method of Compensating for the Increasing Weight of the Gasometer Bell as it Emerges from the Water.—The two counterweights attached to the steel tape (Fig. 158) exactly balance the bell when it is in the water. The small counter weight suspended from the helical spiral (Archimedes spiral) is the difference between the weight of the bell in the water and 100 cm. out of the water.

[1] *Calibration of Gasometer.*—Gasometers of 100 liter capacity or more are usually constructed with two lateral seams. Starting from the shoulder, mark off 5 cm. distances to the bottom of the bell. With a steel tape measure the circumferences at these different heights. (Allowance must be made for the thickness of the seams). The measurements should not vary more than 0.3 cm. An example of a calibration:

The average circumference is 107.05 cm.

The radius would be $\frac{1}{2\pi} \times 107.05 = 17.038$ cm.

Deduct thickness of copper bell, $17.038 - 0.07 = 16.968$ cm. inside radius.
Area of inside $\pi r^2 = 3.1416 \times (16.968)^2 = 904.5$ sq. cm.
Therefore a rise of 1 cm. = 904.5 c.c. or 0.9045 liters.
The logarithm of this factor of the gasometer would be $9.95641 - 10$.

lected, the respiration and pulse rates are recorded at frequent intervals. The duration of the test is determined by the subject's rate of ventilation. As a rule the gasometer is nearly filled at the end of 10 to 12 minutes, although this period may vary from 5 to 15 minutes. Calculation is facilitated by stopping on the minute, the quarter, or the half minute, but care should be taken to turn the valve only on an inspiration.

In a few minutes after the test the gasometer temperature increases; when it is the same as that of the barometer, the barometric pressure and the temperature are recorded. The reading on the steel tape is now recorded to the nearest half millimeter. The difference between this and the first reading (about zero) tells the height in centimeters which the bell has risen. As the bell is exactly counterbalanced in all positions, the enclosed air is under the prevailing barometric pressure. The lower portion of the counterweight is now removed, leaving the air under positive pressure. Samples of the expired air are now collected in gas-sampling bottles.[1]

FIG 159.—HEAD MASK.

Army type of gas mask, held in position by elastic straps and 5-inch rubber sponges to prevent leakage. The incoming air is directed upward toward windows, and opening of expiratory tube is opposite nose and mouth. Pressure on throat may be relieved by drawing away mask and connections which hang from hook over patient's head.

Collection of Air Samples.—In preparation for use, one sets the stopcock so that the spout communicates with the gas chamber (Fig. 160). The bottle is now tilted forward over a small beaker containing 1 per cent sulphuric acid, into which the rubber tube dips. The contained air is forced out by the mercury and when the bottle is tilted backward, acidulated water is drawn into the chamber. The apparatus is again tilted forward, this forces out the air and water. This position is maintained until mercury drops into the beaker (A, Fig. 160). The stopcock is immediately reversed; this leaves the gas chamber and the lower lead of the

[1] The gas sampling bottle (Bailey: *Jour. Biol. Chem.*, 47, 281, 1921) (Fig. 160) consists of parallel glass cylinders communicating at the bottom through a small opening. The cylinders are firmly cemented into a metal case which forms a base. The top of one of the cylinders has a funnel shaped inversion; the other tapers off to a 2 mm. capillary tube to which a 3-way stopcock is fused. The second opening in the stopcock communicates with a capillary tube which bends over the inverted top of the first cylinder. The stopcock terminates in a straight spout of capillary tubing 3 cm. long. The appliance is half filled with mercury, and before use is flushed out with 1 per cent sulphuric acid. A 6 cm. length of $\frac{3}{16}$ inch rubber tubing is permanently attached to the spout.

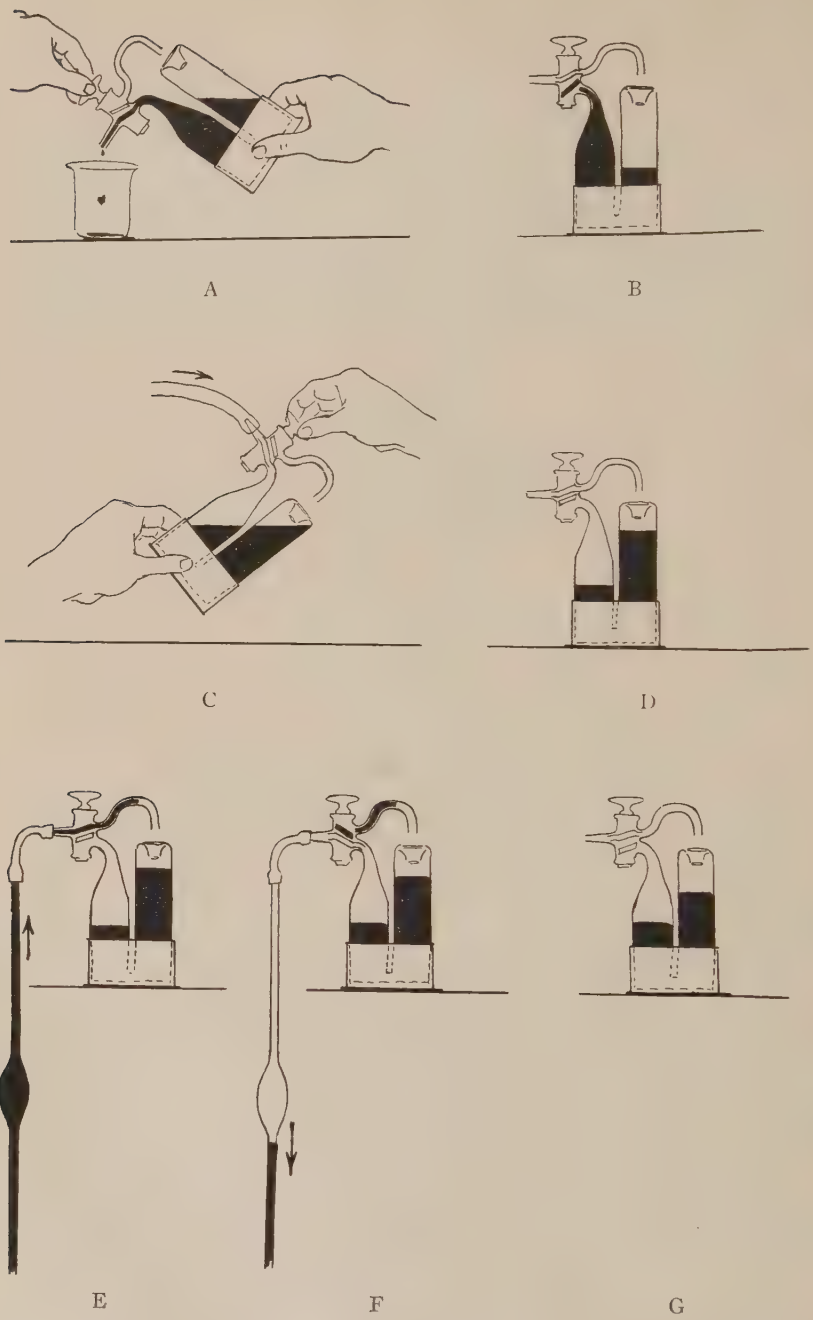


FIG 160.—ILLUSTRATING MANNER OF OPERATING BAILEY GAS SAMPLING BOTTLES.

stopcock filled with mercury (B, Fig. 160) and the spout is in communication with the bent capillary tubing through which the air to be sampled is blown. Enough acidulated water remains in the chamber to saturate the air when the bottle is filled. The lower part of the counterweight is hooked to the wire which suspends the gasometer bell. The enclosed air is now under pressure. About half of the air in the gasometer is allowed to escape through valve J, Fig. 157. The sampling cock (K), which is permanently equipped with a short length of pressure tubing terminating in a glass nozzle, is now opened. The prepared sampling bottle is connected to the nozzle and the expired air forced through the curved tube into the room. The stopcock of the sampling bottle is then reversed, the bottle tilted backward (C, Fig. 160) and the compartment filled with air from the gasometer. The stopcock is returned to its original position and the sample is trapped under pressure in the bottle (D, Fig. 160).

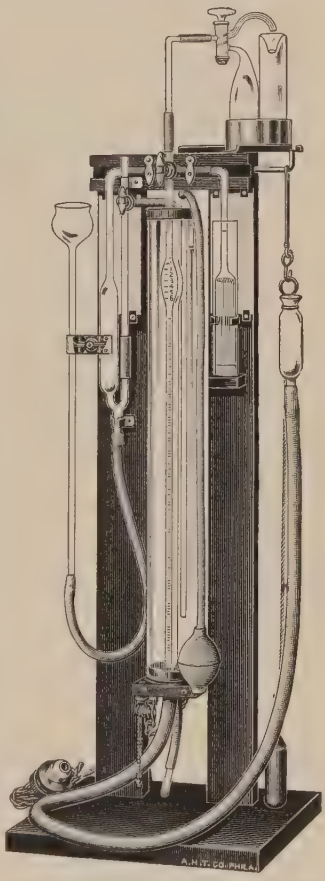


FIG. 161.—BAILEY'S MODIFICATION OF THE HALDANE-HENDERSON GAS ANALYSIS APPARATUS.

Principles of Gas Analysis.—The Haldane method is used in determining the amount of carbon dioxide and oxygen in the expired air. A volume of air is drawn into a graduated burette, where it is saturated with water vapor and measured; the air is then passed back and forth into a potash pipette, where the carbon dioxide is removed; it is returned to the burette and again measured, the loss in volume representing the carbon dioxide. The oxygen is removed in a similar manner by passing the air into a second pipette containing alkaline pyrogallate solution, after which the gas is returned to the burette and measured, this loss in volume representing the oxygen in the sample of air.

In applying this method, several modifications[1] of the original parts have been incorporated in the apparatus, the principal changes being the adoption of Henderson's burette[2] with a four-way stopcock and oxygen absorber. These greatly simplify the assembling and cleansing of the parts and permit one to change the pyrogallate without disconnecting the apparatus.

Description of Apparatus.—The complete apparatus is pictured in Fig. 161. The space behind the water-jacket is closed with ground glass, and behind this is an

[1] Bailey, *Jour. Lab. Clin. Med.*, 6, 657, 1921

[2] Henderson: *Jour. Biol. Chem.*, 33, 31, 1918.

electric light bulb in a chain socket, so that the light can be readily switched on and off during the analysis. The table of corrections for the burette is glued to the support to the right hand side of the ground glass. The frame carries a hook at

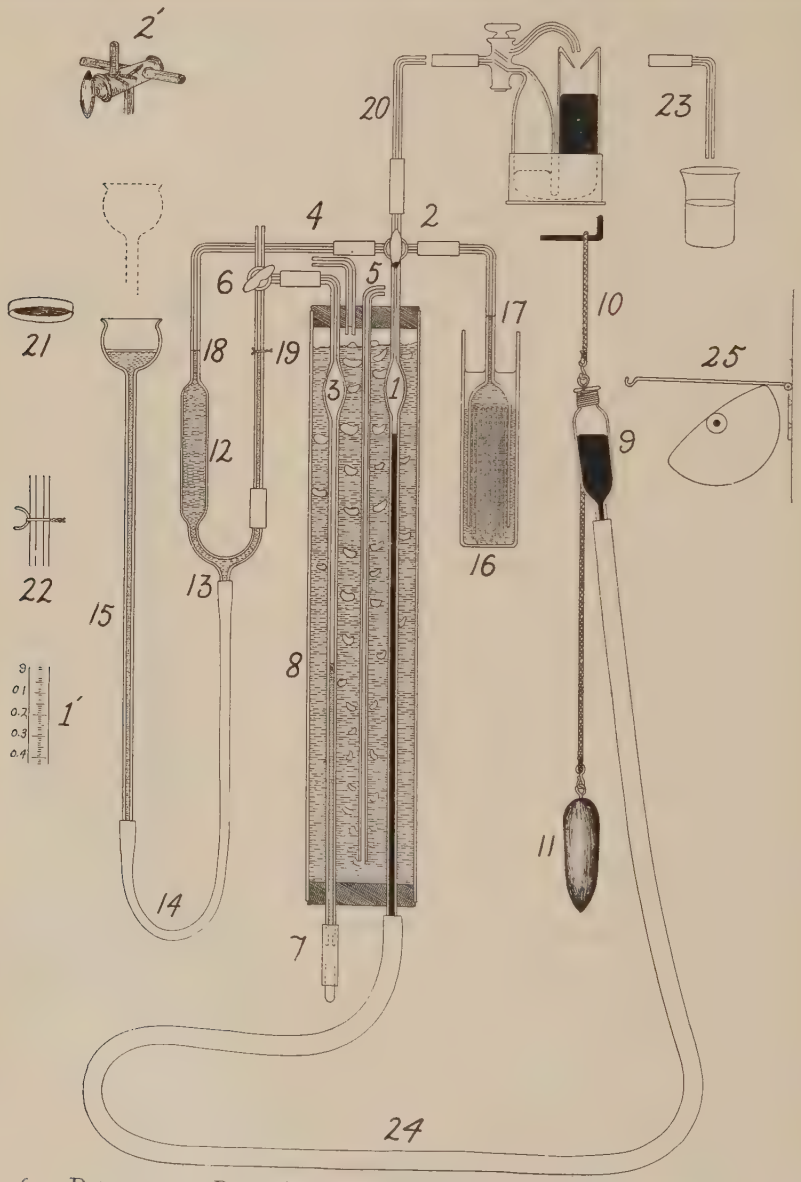


FIG. 162.—DIAGRAM OF BAILEY'S MODIFICATION OF THE HALDANE-HENDERSON GAS ANALYSIS APPARATUS.

the upper right hand corner, from which is suspended the mercury reservoir and its lead counterweight.

The assembled apparatus, is shown diagrammatically in Fig. 162. It consists of a 10 c.c. burette graduated in hundredths (1) having a four-way stopcock at the top

(2); this, along with a control tube of the same volume (3), is enclosed in a water-jacket extending the full length of the burette which is closed at top and bottom by rubber stoppers, through which the burette and control tubes protrude. The upper stopper has two additional perforations, one of which carries a suction tube (4) to the air space above the water; the second carries a tube (5) open at the top and extending to the bottom of the jacket. Air is kept bubbling through the water by suction applied to (4), thus equalizing the temperature of the enclosed burettes. A 6 cm. length of pressure tubing is fastened to the lower end of the control tube. This tube is occluded by a short piece of glass rod when the acidulated water in the control tube is set at the proper level (8).

The lower end of the burette is connected by pressure tubing to a mercury reservoir (9) which is suspended from the hook and counterbalanced by a lead weight (11), which weighs 447 gms. and exactly balances the reservoir and tube filled with mercury when the latter is half-way up the burette. When the reservoir is raised or lowered, there is enough friction between the suspending cord and hook to hold the mercury at any level.

Capillary tubes lead from the top and bottom of the potash pipette (12) respectively to the left lead of the stopcock and to the upper end of the control tube. A 3-way stopcock (6) is placed between the control tube and its manometer. All the connections are made glass to glass and held tight by pressure tubing. The lowest opening in the pipette (13) is connected by sulphur-free seamless rubber tubing (14) to the lower end of the thistle tube (15) which serves as a reservoir for the potassium hydroxide solution. This tube slides through a clamp on the frame, and by raising or lowering the reservoir equal changes of pressure are produced in the burette and in the control tube. The thistle tube is loosely covered to keep it free from dust (21).

The right lead from the stopcock is continuous with a capillary tube which terminates in an inverted bell enclosed in a flat-bottomed tube (16). This bell is loosely filled with lengths of 3 mm. thin walled glass tubing, bevelled on the ends which rest on the bottom of the tube. When in use, this tube holds the pyrogallate covered with a 2 cm. layer of petroleum oil, which protects it from the air. This part of the apparatus constitutes the oxygen absorber.

The capillary tubes leading from the absorbers serve as manometers in adjusting the apparatus. A permanent mark (17) is cut in the tubing of the oxygen absorber 5 cm. above the bell. Sliding markers, as described by Henderson, are conveniently used on the manometer tubes leading from the CO_2 absorber (18, 19). These are readily made by twisting a loop of fine brass wire around the tube, along which is laid a short length of a rubber band. This marker (22) is readily moved to the required position by grasping the ends of the rubber and the twisted wires; the rubber between the wire and the glass holds it firmly in position.

The upper lead from the stopcock is connected by pressure tubing to an elbow of capillary tubing (20), the horizontal part of which is 2½ cm. long and is at a height of 13 cm. above the shelf upon which the sampling bottle rests.

The burette,[1] as supplied by the manufacturers, has a stopcock fused on the lower end. After recalibration this is cut off and the end of the burette smoothed with a fine file under water (not fire-polished).

[1] *Calibration of a Henderson-Haldane-Bailey Burette* —The burette (1, Fig. 162) is thoroughly cleansed with warm cleaning solution, rinsed with distilled water, and dried by sucking air through it. The stopcocks are carefully greased. The burette is then mounted in its glass jacket, a thermometer is enclosed with it. The 4-way stopcock is turned to

Before assembling the apparatus, the glass parts should be thoroughly cleansed with soap and water, then with warm cleaning solution, and finally rinsed in distilled water. All rubber tubing should be scrubbed in soapy water in order to completely remove the talc, and finally rinsed and flushed out with 1 per cent sulphuric acid. In assembling the apparatus, a small amount of vaseline may be used in slipping the rubber over the glass tubing; this makes a very tight joint as it causes the rubber to adhere. Black rubber grease[1] is used on the stopcock.

Procedure for Gas Analysis.—Preliminary.—Fill the water bath with distilled water to within one inch of the upper stopper. Introduce acidulated water into the control tube, setting the level (8) opposite to the 8.9 c.c. graduation on the burette. The water is adjusted to this level by means of the glass plug (7). Remove the plug of stopcock (2). Lower the mercury reservoir (9) until its opening is opposite to the bottom of the water bath, and fill to the neck with mercury. Turn stopcock (6) to exclude atmosphere. Place the thistle tube (15) at the level shown in the diagram and fill the pipette (12) with the dilute solution of potassium hydroxide[2] until the level in the thistle tube is opposite to the mark (18). Superimpose a thin layer of mineral oil to prevent evaporation and replace the cover (21) on thistle tube. Admit potash to manometer (19) by gradually turning stopcock (6).

Fill the oxygen absorber (16) with the alkaline pyrogallate solution to the level of the top of the bell, and superimpose a 2 cm. layer of petroleum oil.

Grease the plug of the stopcock with the black rubber mixture, care being taken that the holes are not occluded. Place the plug in position and turn with gentle pressure until the burette is in communication with the intake tube (20). The black mark on the stopcock handle indicates the lead which is in communication with the burette. Attach glass elbow (23) to the intake tube (20) so that the former points downward, and dips into dilute sulphuric acid contained in a beaker

communicate with one of the lateral leads. The lower stopcock is opened. A 1-meter length of pressure tubing carrying a separatory funnel containing mercury is now attached to the nozzle of the lower stopcock. Mercury is slowly admitted to the burette by gently raising the funnel. This is continued until mercury appears in the side lead. The lower stopcock is now closed and the filling apparatus removed. Care is taken that bubbles of air are not left in the burette, and that the mercury reaches to the tip of the nozzle, which must have a very small bore. A weighing bottle is carefully tared. A balance is prepared for weighing. The room temperature is kept constant. The 4-way stopcock is turned to communicate with the upper lead, this cuts off the mercury column below the plug and leaves it exposed to the air. The weighing bottle is held under the lower stopcock so that the nozzle touches the bottom. By gently turning the lower stopcock mercury is allowed to escape from the burette until the meniscus is slightly below the 7 c.c. mark. Gently tap the burette to make the meniscus stand freely. Read the volume and weigh the bottle and its contents. Another portion of mercury, about 0.2 c.c., is added to the bottle in a similar manner and the volume and the total weight recorded as before. This is repeated at intervals for the length of the burette. The last reading must be slightly less than 10.0 c.c. We now have the volumes as read on the burette and the corresponding weights of mercury. The density of the mercury is determined by dividing the total weight of mercury in the bottle by the last reading. Now, if we divide the different weights by the density we get the true volumes corresponding to the burette readings. A curve of the differences is plotted on millimeter paper and a table of corrections is made from this. As a check, the calibration should be repeated. A good burette should have a correction of not over 0.003 c.c.

[1] This is prepared by heating together equal parts of finely cut black rubber tubing, and lanolin, until the rubber has completely dissolved.

[2] The absorbing solutions are conveniently prepared from a stock saturated solution of potassium hydroxide. For use in the potash pipette this is diluted to a sp. gr. of 1.15. The alkaline pyrogallate is made by diluting the stock alkali to sp. gr. 1.55 and adding 10 gm. of pyrogallic acid per 100 c.c.

held beneath the tube. The burette is now flushed out with the acid by raising and lowering the mercury reservoir (9). The burette is then emptied by raising the reservoir until a few drops of mercury fall into the beaker. Remove the glass elbow (23).

The tube (4) is connected with a suction pump and an air current kept bubbling through the water-jacket. Before one can use the apparatus, it is necessary to absorb the carbon dioxide and oxygen from the air which fills the capillary tubes between the fluids and the stopcock. This is accomplished by drawing about 10 c.c. of air into the burette. Now make a quarter-turn of the stopcock clockwise at the same time slightly raising the mercury reservoir (9) so as to put the air under positive pressure and prevent the possible sucking of pyrogallate into the stopcock and burette. Absorption is brought about by alternately raising and lowering the reservoir (9). This pushes the gas back and forth between the burette and absorber, and brings it constantly in contact with new surfaces of fluid. In doing this, the mercury should not be carried above or below the bulb of the burette. This procedure is repeated 18 to 20 times. The reservoir is then carefully lowered until the pyrogallate enters the manometer tube. Now connect the burette with the potash pipette. This is accomplished by making a full half-turn of the stopcock *clockwise* at the same time slightly raising the reservoir (9), as before, to prevent sucking the alkali into the stopcock and burette. The air is now washed back and forth several times and returned to the burette by lowering the reservoir (9). Do not let the potash reach the elbow above (18).

The burette is again put in communication with the oxygen absorber by making a full half-turn of the stopcock in the opposite direction to the hands of a clock, taking care to raise the reservoir slightly as before. This procedure is repeated several times until the readings of the burette remain constant.

Preparatory to reading the volume, the pyrogallate is brought to its mark (17) on the manometer; the burette is then connected with the potash pipette by a half-turn of the stopcock clockwise with the usual slight raising of the mercury reservoir. The level in the potash manometer is now set by rolling the cord back or forth over the hook; at the same time the volume of the control tube is returned to normal by raising or lowering the thistle-tube (15). In this way changes of volume, due to temperature changes, are overcome by equally increasing or decreasing the pressure exerted on the two volumes of gas by the head of fluid in the thistle-tube. In order to be sure that free communication exists between the burette and the manometers, momentarily pinch the rubber tubing and observe that the levels in the manometers balance freely with the mercury. A drop of fluid or grease in the stopcock may interfere with the free transmission of pressure and lead to gross errors in the analysis. These obstructions, if present, can usually be dislodged by repeatedly pinching the tubing. The levels being properly set at their marks on the manometer tubes, switch on the light which is placed at the back of the water-jacket; read the burette to the thousandth part of a cubic centimeter by means of a magnifying glass, placing the eye so that the graduation mark, below the bottom of the mercury meniscus, and its reflection from the mercury are directly in line; the location of the top of the meniscus between the hundredths divisions is then estimated in tenths. A small amount of acidulated water inside the burette is necessary to insure that the air is saturated with water vapor. If one flushes the burette with the dilute acid immediately before each test, sufficient moisture remains to bring about this saturation.

When not in use, the nitrogen remaining after the last analysis is kept in the oxygen absorber; the stopcock is turned so that the burette communicates with the intake-tube (20); the potash reservoir (15) is lowered below (18). Open stopcock (6) to communicate with the atmosphere. The apparatus can be left safely in this manner, without the danger of alkali being drawn into the burette as a result of temperature changes.

Preparation for Test.—Start the suction at (4). Close stopcock (6) to exclude the atmosphere. Attach tube (23) to tube (20), dip the lower end of (23) into a beaker of 1 per cent H_2SO_4 solution and wash burette by lowering and raising reservoir (9). (Do not lower the mercury below the bottom of the water jacket as dirt may be carried up into the burette). Empty the burette by raising the reservoir (9) above the tube (23) and lowering at once when mercury begins to drop into the beaker, this leaves some acidulated water floating on the mercury in the burette. Remove tube (23) and attach sampling bottle, making a close glass connection.

Setting the Level (17).—Raise the reservoir (9) until the mercury passes into tube (20) above the stopcock; a bubble of air may stick to the grease in the stopcock and not be dislodged by the acidulated water. The mercury dislodges this bubble which attaches itself to the mercury meniscus; by a slight up and down motion of (9) the bubble is dislodged, leaving about 1 cm. length of acidulated water between the air and the mercury meniscus. The mercury is then carefully lowered until the mercury meniscus appears just below the stopcock; holding the mercury at this level, stopcock (2) is given one-fourth turn clockwise to communicate with the oxygen absorber (16) and upon lowering the reservoir (9) the excess nitrogen is drawn into the burette until the pyrogallate stands at the level (17). The stopcock is then given a half turn clockwise and the nitrogen gently pushed into the potash pipette (12) by raising the reservoir (9). Avoid pushing mercury into stopcock.

Setting the Potash and Control Tube Manometers.—Lower the reservoir (9) until the potash solution reaches the level (18). Open stopcock (6). Set the thistle tube level opposite to 18. By means of reservoir (9), adjust the meniscus at (18), and holding it at this level turn the stopcock (2) *clockwise* to communicate with the intake (20). Gently pinch the tube (14) and observe that the fluid moves freely in the control manometer; close stopcock (6), excluding the atmosphere and leaving the control tube in communication with the manometer. Pinch tube (14) and if the fluid bounces freely the passage is known to be unobstructed. Now set the sliding marker (19) to record the position of the meniscus in the control manometer. The apparatus is now ready to receive a sample of air for analysis.

The burette and intake-tube are completely emptied of air by raising the mercury reservoir (9); this causes the mercury to rise in the burette, forcing the air and most of the acidulated water before it. When the mercury has passed into the curved tube as shown in (E, Fig. 160), the stopcock of the bottle is given a half turn, the reservoir lowered, and the expired air passes into the burette as shown in (F, Fig. 160). (In this manipulation the reservoir is grasped in the hand and raised slightly forward from the hook, leaving the cord in position.) The sample is washed back into the bottle and again drawn into the burette by raising and lowering the mercury reservoir. This air is considerably compressed, and in order to take a sample which can be measured at atmospheric pressure, one

sets the mercury meniscus at about 9.2 c.c. The stopcock of the bottle is then given a quarter of a turn, and the reservoir lowered until the meniscus in (9) is level with the meniscus in the burette. Next, the four-way stop cock is carefully given a quarter-turn anticlockwise and the levels in the manometer tubes (18) (19) rapidly reset by manipulating the thistle-tube (15) and the mercury reservoir (9). The volume of gas in the burette is now read to a thousandth part of a c.c. in the manner already described.

The carbon dioxide is absorbed by alternately raising and lowering the mercury reservoir, not permitting the mercury to rise above, or pass below, the bulb of the burette. After passing the gas, in this manner, eight or nine times one resets the levels (18 and 19) and reads the burette. The gas is again passed five or six times and the volume recorded. If the volumes do not agree within 0.002 c.c., the procedure is repeated until check readings are obtained.

To absorb the oxygen, one gently lowers the mercury reservoir (9) until the potash is within an inch of the bend above the mark (18). The stopcock (2) is then given a half-turn anticlockwise, at the same time slightly raising the mercury reservoir to avoid the danger of sucking pyrogallate into the stopcock and burette. The gas is passed about eighteen times into the oxygen absorber. It is then passed twice into the potash pipette, in order to wash out the oxygen which was left in the capillary tube and again passed into the oxygen absorber an additional nine or ten times. This combined procedure is repeated twice, the pyrogallate meniscus reset (17), the burette thrown into communication with the potash pipette, the levels (18 and 19) readjusted, and the volume read on the burette. Absorption should be continued until check readings are obtained.

These three volumes are now corrected according to the recalibration of the burette. The difference between the second and first readings represents the volume of carbon dioxide in the sample. The difference between the third and second readings represents the volume of oxygen in the sample. The percentages of these gases are now calculated.

When finished analyzing, the nitrogen is stored in the oxygen absorber (16), the thistle tube is lowered, and the stopcocks (6 and 2) are opened to the atmosphere.

Analysis of Outdoor Air.—As has already been stated, the atmosphere is of very constant composition. Advantage is taken of this fact is testing the accuracy of gas analysis apparatus. Outdoor air should be analyzed at least once a week, the results for carbon dioxide should be within 0.02 per cent and for oxygen 0.03 per cent of the standard values. (See p. 498.) The routine analyses of the expired air provide additional checks. In each case duplicate analyses are made using different apparatus. If the results are not within the permissible error, a third analysis is carried out.

CHAPTER XXII

MILK

Milk is the most satisfactory individual food material elaborated by nature. It contains the three nutrients, protein, fat, and carbohydrate and inorganic salts in such proportion as to render it a very acceptable dietary constituent. Its dietary value is also enhanced by the presence of the four *vitamins*, vitamin A, vitamin B, vitamin C and the *antirachite factor*. Milk would be an ideal food for children as well as adults were it not for its low content of vitamin B[1] and of iron together with a deficiency of organic substances essential for hemoglobin

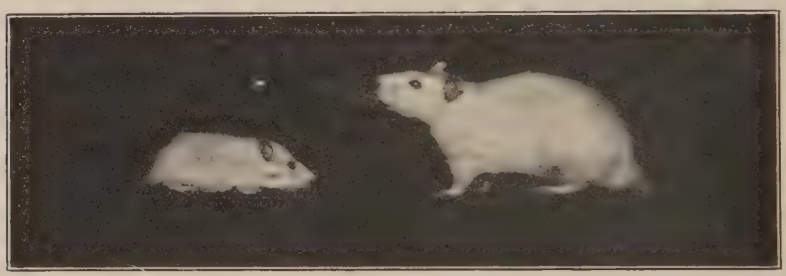


Fig. 163.—The Dietary Efficiency of Milk. The rats were twin sisters. The first received bread and apple, the second bread and milk. The bread and milk diet led to rapid growth. The bread and apple diet was deficient in protein, calcium, phosphorus and vitamin A. (Sherman, Rouse, Allen and Woods: *Journal of Biological Chemistry*, 46, 503, 1921.) See experiment on Dietary Efficiency of Milk, Chapter XXXII.

formation and for reproduction.[2] The dietary efficiency of milk is illustrated in Fig. 163. Sherman and Campbell[3] have shown, with rats, an increase of the proportion of milk in an already adequate diet to improve the general nutrition. Milk is a specific product of the secretory activity of the mammary gland. It contains, as the principal solids, *olein*, *palmitin*, *stearin*, *butyrin*, *casein*, *lactalbumin*, *lacto-globulin*, *lactose*, *phosphates* of *calcium*, *potassium*, and *magnesium*, *citrates* of *sodium* and *potass um*, *chloride* of *calcium*, *iron* and *vitamins*. It also contains at least traces of lecithin, cholesterol, urea, creatine, creatinine, and the tri-glycerides of caproic, lauric and myristic acids. The calcium phosphate of milk is the neutral calcium phosphate, $CaHPO_4$.[4]

[1] Osborne and Mendel: *Jour. Biol. Chem.*, 41, 515, 1920.
[2] Hart, Steenbock, Elvehjem, and Waddell: *Jour. Biol. Chem.*, 65, 67, 1925.
Mattill, Carman, and Clayton: *Jour. Biol. Chem.*, 61, 729, 1924.
[3] Sherman and Campbell.: *Jour. Biol Chem.*, 60, 51, 1924.
[4] Van Slyke and Bosworth: *Jour. Biol. Chem.*, 20, 135, 1915.

According to Osborne and Wakeman[1] milk contains two *phosphatides*, one being probably *steryloleyl-lecithin*. These same investigators[2] have also shown milk to contain an *alcohol-soluble* protein.

The presence of the vitamin Fat-soluble A *in butter fat* was first demonstrated by McCollum and Davis and by Osborne and Mendel.[3] For discussion of vitamins, see Chapter XXXII).

Summer milk has a higher vitamin content than winter milk.[4]

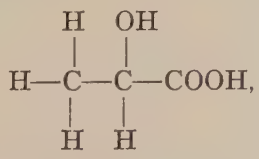
The preparation of milk in the form of a *powder* has become an important industry.

By passing milk through a special form of earthenware filter Van Slyke and Bosworth[5] have obtained a separation of the constituents in milk which are in true solution from those insoluble in water or in suspension. The soluble constituents and the water constitute the *milk serum*. Their classification of milk constituents in a slightly modified form follows:

CONSTITUENTS OF MILK

In true solution in milk serum.	Partly in solution and partly in suspension or colloidal solution	Entirely in suspension or colloidal solution.
1. Lactose.	1. Albumin.	1. Fat.
2. Citric acid.	2. Inorganic phosphate.	2. Casein.
3. Potassium.	3. Calcium.	3. Vitamin A.
4. Sodium.	4. Magnesium.	
5. Chlorine.		
6. Vitamin B.		
7. Vitamin C.		

Fresh milk, *both human* and *cow's*, is amphoteric in reaction to litmus and *acid to phenolphthalein* (pH 6.7). The acidity is believed to be due in part at least to *soluble acid phosphates*. Upon standing for a sufficiently long time, unsterilized milk *sours, i.e.*, it becomes strongly acid in reaction to litmus due to the production of the optically inactive fermentation lactic acid,



from the lactose contained in it. This is brought about through bacterial activity. The white color is imparted to the milk partly

[1] Osborne and Wakeman: *Jour. Biol. Chem.*, 21, 539, 1915; 28, 1, 1916.
[2] Osborne and Wakeman: *Jour. Biol. Chem.*, 33, 243, 1918.
[3] McCollum and Davis: *Jour. Biol. Chem.*, 15, 167, 1913; Osborne and Mendel: ibid., 15, 311, 1913; 24, 37, 1916 (previous references cited in this article).
[4] Hart, Steenbock and Ellis: *Jour. Biol. Chem.*, 42, 383, 1920. Hess, Unger and Supplee: *Jour. Biol. Chem.*, 45, 229, 1920. Dutcher, Kennedy and Eckles: *Science*, 52, December 17, 1920.
[5] Van Slyke and Bosworth: *Jour. Biol. Chem.*, 20, 135, 1915.

through the fine emulsion of the fat and partly through the medium of the caseinogen in solution. The specific gravity of milk varies somewhat, the average being about 1.030. Its freezing-point is about −0.56°C.

This *lactic acid fermentation* may be brought about by *Bact. lactis acidi* and other microorganisms. Certain putrefactive bacteria in the human intestines may also cause lactic acid fermentation. The chemical changes in lactic acid fermentation may be indicated thus:

$$\underset{\text{Lactose.}}{C_{12}H_{22}O_{11}} + H_2O \rightarrow \underset{\text{Galactose.}}{C_6H_{12}O_6} + \underset{\text{Glucose.}}{C_6H_{12}O_6}$$

$$\underset{\substack{\text{Galactose}\\ \text{or Glucose.}}}{C_6H_{12}O_6} \rightarrow 2\underset{\text{Lactic Acid}}{C_3H_6O_3}$$

Fresh milk does not coagulate on being boiled but a film consisting of a combination of casein and calcium salts forms on the surface. If the film be removed, thus allowing a fresh surface to come into

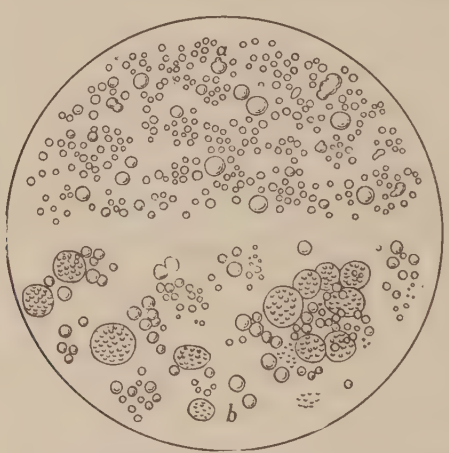


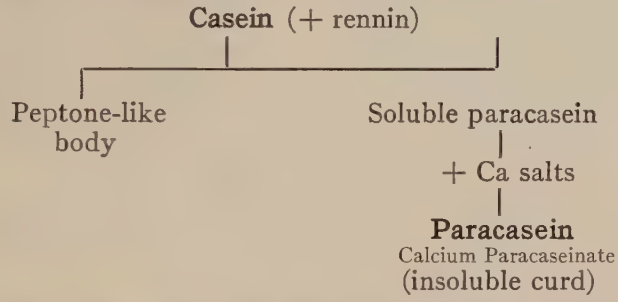
Fig. 164.—Normal Milk and Colostrum.
a, Normal milk; *b*, Colostrum.

contact with the air, a new film will form indefinitely upon the application of heat. Surface evaporation and the presence of fat facilitate the formation of the film, but are not essential (Rettger[1]). As Jamison and Hertz[2] have shown, a similar film will form on heating any protein solution containing fat or paraffin. If the milk is of a pronounced acid reaction, through the inception of lactic acid fermentation, or from any other cause, no film will form when heat is applied, but instead a true coagulation will occur. When milk is boiled certain changes occur in its odor and taste. These changes, according to Rettger,[3] are due

[1] Rettger: *American Journal of Physiology*, 7, 325, 1902.
[2] Jamison and Hertz: *Journal of Physiology*, 27, 26, 1902.
[3] Rettger: *American Journal of Physiology*, 6, 450, 1902.

to a partial decomposition of the milk proteins and are accompanied by the liberation of a volatile sulphide, probably hydrogen sulphide.

The milk-curdling enyzmes of the gastric and the pancreatic juice have the power of splitting the casein of the milk, through a process of hydrolytic cleavage, into *soluble paracasein* and a *peptone-like* body.[1] This soluble paracasein then forms a combination with the soluble calcium salts of the milk and an *insoluble* curd of *paracasein* results. The clear fluid surrounding the curd is known as *whey*. This action of rennin may be represented by the following scheme:



There is still considerable confusion of terms when different authorities discuss milk proteins and the action of milk curdling enzymes upon

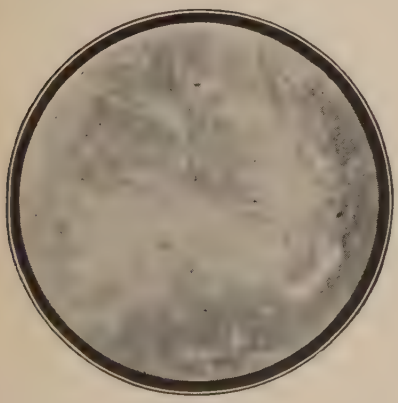


FIG. 165.

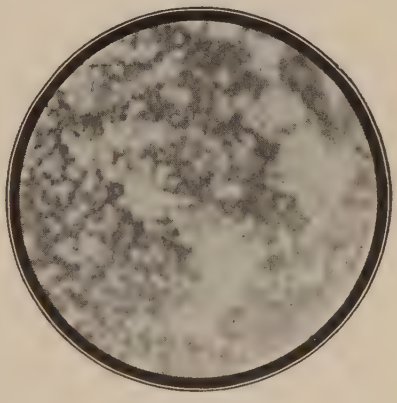


FIG. 166.

FIG. 165.—CURD OF HUMAN MILK 5 MINUTES AFTER INGESTION OF 75 C.C. MILK (Beginning of curd formation, ½ actual size).[2]

FIG. 166.—CURD OF HUMAN MILK 10 MINUTES AFTER INGESTION OF 75 C.C. MILK Maximum curd formation, ½ actual size).[2]

them. The English scientists[3] quite uniformly call the principal protein of milk *caseinogen* whereas the insoluble curd formed by rennin is

[1] Bosworth (*Am. Jour. Dis. Child.*, 22, 193, 1921) however, finds that the casein is split into two equal molecules of paracasein.

[2] Unpublished photographs from the thesis of Dr. Robert A. Lichtenthaeler.

[3] Halliburton: *Journal of Physiology*, 11, 448, 1900.

termed *casein*. On the other hand, the Germans and many Americans give the name *casein* to the milk protein and *paracasein* to the product

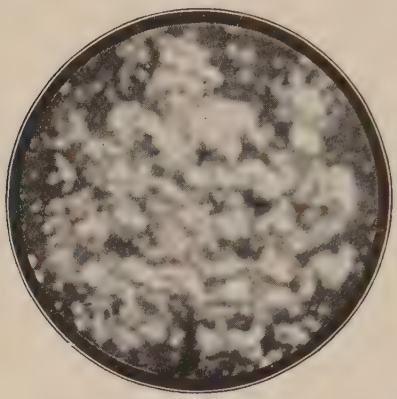


Fig. 167

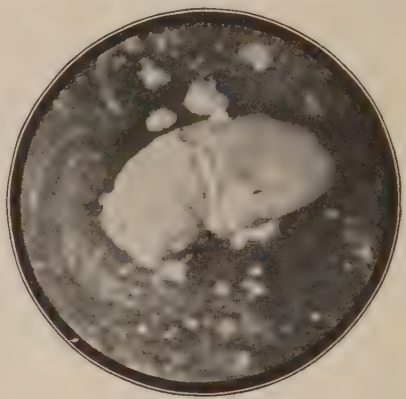


Fig. 168.

Fig. 167.—Curd of Cow's Milk Regurgitated 10 Minutes after Ingestion of 500 c.c. Whole Milk. (Curds ½ actual size).[1]

Fig. 168.—Curd of Cow's Milk Regurgitated 25 Minutes after Ingestion of 500 c.c. Whole Milk. (Curds ½ actual size).[1]

Figs. 165 to 168.—Photographs Representing Typical Curds From Human and Cow's Milk.

of the action of rennin upon this protein. The confusion of terms may be represented thus:

English.		*German.*
Caseinogen.	=	Casein.
Casein.	=	Paracasein.

The most important difference between human milk and cow's milk is in the protein content, although there are also differences in the carbohydrate and ash and likewise striking biological differences difficult to define chemically. It has been shown that the casein of human milk differs from the casein of cow's milk in being more difficult to precipitate by acid or coagulate by gastric rennin. The casein curd (paracasein) also forms in much looser and more flocculent manner than that from cow's milk and is for this reason much more easily digested than the latter. (For illustrations of milk curds, see Figs. 165 to 168, pages 533 and 534.) Both human and cow's milk contain important non-nitrogenous substances of an unknown character. Human milk contains the greater quantity of these substances.[2]

Non-protein nitrogen, *urea*, *uric acid*, *amino acids* and *creatinine* are present in normal human milk in approximately the same concentration as in normal human blood[3] (See Chapter XVIII). The cholesterol content of human milk usually varies with the fat content, averaging from 10 to 30 mg. per 100 c.c.[4]

[1] Bergeim, Evvard, Rehfuss and Hawk: *Am. Jour. Physiol.*, 48, 411, 1919.
[2] Meigs and Marsh: *Jour. Biol. Chem.*, 16, 147, 1913.
[3] Denis, Talbot and Minot; *Jour. Biol. Chem.*, 39, 47, 1919.
[4] Denis and Minot: *Jour. Biol. Chem.*, 36, 59, 1918.

The relative composition of human and cow's milk is shown in the following table which embraces data reported by Meigs and Marsh.

COMPOSITION OF MILK (PER CENT OF WHOLE MILK) NORMAL VARIATIONS FROM BEGINNING OF SECOND MONTH OF LACTATION

Constituent	Cow	Human
Water (Avg.)	87.0	87.5
Solids (Avg.)	13.0	12.5
Protein	4–2.5[1]	1.5–0.7
Fat	2–4	2–4
Sugar	3.5–5[1]	6–7.5
Ash	0.6–0.7	0.2–0.3

The above data indicate that *human milk contains less protein, more sugar and much less ash than cow's milk.* The percentage composition of human milk at different periods is represented in the following table.[2]

PERCENTAGE COMPOSITION OF HUMAN MILK BY PERIODS

Period	Fat	Sugar	Protein	Casein	Albumin	Ash	Total solids
Colostrum (1–12 days)	2.83	7.59	2.25	.....	.....	0.31	13.4
Transition (12–30 days)	4.37	7.74	1.56	.....	.....	0.24	13.4
Mature (1–9 mos.)	3.26	7.50	1.15	0.43	0.72	0.21	12.2
Late (10–20 mos.)	3.16	7.47	1.07	0.32	0.75	0.20	12.2

The composition of the ash of milk is shown in the following table reported by Holt, Courtney and Fales.[2]

PERCENTAGE COMPOSITION OF THE ASH OF MILK

	CaO	MgO	P_2O_5	Na_2O	K_2O	Cl
Human milk	23.3	3.7	16.6	7.2	28.3	16.5
Cow's milk	23.5	2.8	26.5	7.2	24.9	13.6

[1] Protein starts high and *decreases* whereas sugar starts low and *increases.*
[2] Holt, Courtney and Fales: *Am. Jour. Dis. Children,* 10, 229, 1915.

It will be observed that the composition of the ash of the two varieties of milk is about the same except for phosphorus. The higher phosphorus content in the case of cow's milk is due principally to the fact that the milk contains a higher percentage of *casein* or *phosphoprotein*. It should be borne in mind that cow's milk contains on the average over *three times* as much ash as human milk. Therefore unless cow's milk has been diluted with more than twice its volume, there is still present as high a concentration of the inorganic constituents as are present in normal human milk. Hence there is no necessity for the addition of any of these constituents in infant feeding.

In view of the demonstrated food value of *dried* cow's milk the recent announcement[1] that *dried human milk* is of service in infant feeding assumes particular importance.

Interesting data relative to the composition of milk from various sources may be gathered from the following table which was compiled mainly from the results of investigations by Pröscher[2] and by Abderhalden[3] in Bunge's laboratory. It will be noted that the composition of the milk varies directly with the length of time needed for the young of the particular species to double in weight.

Species	Period in which weight of the newborn is doubled (days)	100 Parts of milk contain			
		Proteins	Salts	Calcium	Phosphoric acid
Man	180	1.6	0.2	0.033	0.047
Horse	60	2 0	0.4	0.124	0.131
Cow	47	3.5	0.7	0.160	0.197
Goat	22	3.7	0.8	0.197	0.284
Sheep	15	4 9	0 8	0.245	0.293
Pig	14	5.2	0.8	0.249	0.308
Cat	9.5	7.0	1.0	.....	.....
Dog	9	7.4	1.3	0.455	0.508
Rabbit	6	10.4	2.5	0.891	0.997

The secretion of the mammary glands of the newborn of both sexes is called "witches' milk." The name is centuries old and evidently refers to the mystery of the useless secretion. Basch[4] has suggested that this secretion of "witches' milk" is brought about by the passage of *hormones* (see Chapter XX on Endocrine Organs) from the blood of the mother to the fetus.

Lactose, the principal carbohydrate constituent of milk, is an important member of the disaccharide group. It occurs only in milk, except

[1] Emerson, P. W.: *Am. Jour. Dis. Child.*, 30, 769, 1925.
[2] Pröscher: *Zeit. f. physiol. Chemie*, 24, 285, 1898.
[3] Abderhalden: *Ibid.*, 26, 487, 1899; and 27, pp. 408 and 457, 1899
[4] Basch: *Münch. med. Woch.*, 58, 2260, 1911.

as it is found in the urine of women during pregnancy, during the nursing period, and soon after weaning; it also occurs in the urine of normal persons after the ingestion of a very large amount of lactose in the food. It is not derived directly from the blood, but is a specific product of the cellular activity of the mammary gland. It has strong reducing power, is dextro-rotatory and forms an osazone with phenylhydrazine. Lactose is *not* fermentable by the ordinary baker's yeast. For changes which lactose undergoes in *lactic acid fermentation* see page 89. The crystalline form of lactose is shown in Fig. 169.

Casein, the principal protein constituent of milk, belongs to the group of phosphoproteins and contains 0.7 per cent of phosphorus.[1] It has acidic properties and combines with bases to produce salts.[2]

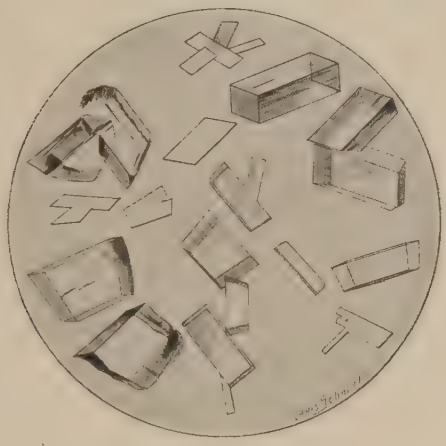


FIG. 169.—LACTOSE.

It is probably present in cows' milk in the form of *neutral calcium caseinate* (Casein Ca_4) and in human milk mainly as potassium caseinate.[3] It is not coagulable upon boiling and is precipitated from its neutral solution by certain metallic salts as well as upon saturation with sodium chloride or magnesium sulphate. Its acid solution is precipitated by mineral acid. (For molecular weight see p. 114.)

Lactalbumin and lacto-globulin, the protein constituents of milk, next in importance to casein, closely resemble serum albumin and serum globulin in their general properties.

Butter (milk fat) consists in large part of *olein* and *palmitin*. Stearin, butyrin, caproin and traces of other fats are also present.

[1] Bosworth and Van Slyke: *Jour. Biol. Chem.*, 19, 67, 1914.
[2] Van Slyke and Bosworth: *Jour Biol. Chem.*, 14, 207–227, 1914.
[3] Van Slyke and Bosworth: *Jour. Biol. Chem.*, 20, 135, 1915; Bosworth: *Am. Jour. Dis. Child.*, 22, 193, 1921.

Of the known vitamins butter contains fat-soluble A the growth vitamin and a smaller amount of vitamin D (the antirachitic factor.)[1] When butter becomes rancid through the cleavage of certain of its constituent fats by bacteria the odors of caproic and butyric acids are in evidence.

The pigment of the fat of cow's milk is made up of *carotin* and *xanthophylls*. The principal pigment is carotin, an unsaturated hydrocarbon pigment which is widely distributed in plants.[2] The pigment of the fat of *human* milk is made up of carotin and xanthophylls in about equal proportions. Carotin is also probably the pigment of human fat. The pigment of body fat, blood serum, corpus luteum and skin secretions of the cow is principally carotin.

Colostrum is the name given to the product of the mammary gland secreted for a short time before parturition and during the early period of lactation (see Fig. 164, page 532). It is yellowish in color, contains more solid matter than ordinary milk, and has a higher specific gravity (1.040–1.080). The most striking difference between colostrum and ordinary milk is the high percentage of globulin in the former. This globulin appears to be identical with the euglobin of blood serum and seems to furnish this protein to the blood of the infant, which blood at birth is very low in globulin. The protective antibodies of the blood are associated with this globulin fraction.[3] This peculiarity in the protein content is responsible for the coagulation of colostrum upon boiling.

Such enzymes as lipase, amylase, galactase, catalase, oxidases, peroxidases, and reductases have been identified in milk, but not all of them in milk of the same species of animal.

Among the principal preservatives used in connection with milk are formaldehyde, hydrogen peroxide, boric acid, borates, benzoic acid, salicylic acid, and salicylates. The use of milk preservatives is illegal in most states.

Experiments on Milk

1. Reaction.—Test the reaction of fresh cow's milk to litmus, phenolphthalein and Congo red.

2. Biuret Test.—Make the biuret test according to directions given on page 164.

3. Microscopical Examination.—Examine fresh whole milk, skimmed or centrifugated milk, and colostrum under the microscope. Compare the microscopical appearance with Fig. 164, page 532.

[1] See Chapter XXXII.

[2] Palmer and Eckles: *Jour. Biol Chem.*, 17, 191, 1914.

[3] Lewis and Wells: *Jour. Am. Med. Assn.*, 78, 863, 1922; Howe: *Jour. Biol. Chem.*, 49, 115, 1921.

4. Specific Gravity.—Determine the specific gravity of both whole and skimmed milk (see page 543). Which possesses the higher specific gravity? Explain why this is so.

5. Film Formation.—Place 10 c.c. of milk in a small beaker and boil a few minutes. Note the formation of a film. Remove the film and heat again. Does the film now form? Of what substance is this film composed? The biuret test was positive; why do we not get a coagulation here when we heat to boiling?

6. Coagulation Test.—Place about 5 c.c. of milk in a test-tube, acidify slightly with dilute acetic acid and heat to boiling. Do you get any coagulation? Why?

7. Action of Hot Alkali.—To a little milk in a test-tube add a few drops of potassium hydroxide and heat. A yellow color develops and gradually deepens into a brown. To what is the formation of this color due? (See Moore's Test, page 74.)

8. Test for Chlorides.—To about 5 c.c. of milk in a test-tube add a few drops of very dilute nitric acid to form a precipitate. Filter off this precipitate and test the filtrate for chlorides. Does milk contain any chlorides?

9. Guaiac Test.—To about 5 c.c. of water in a test-tube add 3 drops of milk and enough alcoholic solution of guaiac (strength about 1 : 60)[1] to cause turbidity. Thoroughly mix the fluids by shaking and observe any change which may gradually take place in the color of the mixture. If no blue color appears in a short time, heat the tube gently below 60°C. and observe whether the color reaction is hastened. In case a blue color does not appear in the course of a few minutes, add hydrogen peroxide or old turpentine, drop by drop, until the color is observed.

Fresh milk will frequently give this blue color when treated with an alcoholic solution of guaiac without the addition of hydrogen peroxide or old turpentine. Those milks which respond positively, fail to do so after boiling 15–20 seconds. What substances beside milk respond to this test? See discussion on page 343.

10. Differentiation of Human and Cow's Milk (Modification of Bauer's Test).[2]—Introduce 2 c.c. of fresh human milk into a 50 c.c. test-tube and 2 c.c. of fresh cow's milk into another similar tube. Add to the contents of each tube 1 drop of a 0.25 per cent aqueous solution of nile-blue sulphate (Grübler). Shake the tubes gently and permit them to stand undisturbed for 10–30 minutes. The milk assumes a bluish cast in each case. At the end of the 10-minute interval add 10 c.c. of ether to the contents of each tube and shake very thoroughly for one minute. The ether extracts the pigment from the human milk, leaving the milk white. In the case of cow's milk the ether does not extract the dye and the milk remains bluish in color.

11. Tests to Differentiate between Raw Milk and Heated Milk.—(*a*) *Tricresol Peroxidase Reaction* (*Kastle*).—The peroxidase reaction of

[1] Buckmaster advises the use of an alcoholic solution of guaiaconic acid instead of an alcoholic solution of guaiac resin. Guaiaconic acid is a constituent of guaiac resin.

[2] Bauer: *Monatssch. f. Kinderheil.*, 11, 474, 1912–13.

milk is founded upon the fact that small amounts of *raw* milk will induce the oxidation of various leuco compounds by hydrogen peroxide This reaction has been used in a practical way as the most convenient means of differentiating between *raw milk* and *heated milk*. Many substances have been employed for this purpose, *e.g.*, guaiac, paraphenylenediamine, ortol, amidol, etc. Kastle has found that a dilute solution of "tricresol"[1] acts as a sensitizing agent in the peroxidase reaction and offers the following test which is based upon this fact.

Procedure.—To 2–5 c.c. of raw milk in a test-tube add 0.1–0.3 c.c. of M/10 hydrogen peroxide and 1 c.c. of a 1 per cent solution of "tricresol." A slight though unmistakable yellow color will be observed to develop throughout the solution. Repeat the test using milk which has been boiled or heated to 80°C. for 10–20 minutes and cooled, and note that no yellow color is produced.

The color reaction in the case of the raw milk probably results from the oxidation of the cresols by the hydrogen peroxide. The first product of this oxidation[2] then oxidizes the leuco compound, when such is present, and causes the color observed.

(*b*) *Benzidine Peroxidase Reaction* (*Wilkinson and Peters*).[3]—To 10 c.c. of the milk to be tested add 2 c.c. of a 4 per cent alcoholic solution of benzidine, sufficient acetic acid to coagulate the milk (usually 2–3 drops) and finally 2 c.c. of a 3 per cent solution of hydrogen peroxide. Raw milk yields an immediate blue color. In adding the peroxide it is best to permit it to flow slowly down the wall of the vessel containing the mixture instead of allowing it to mix with the milk. Milk which has been heated to 78°C. or above remains unchanged.

The reduction of the CO_2 content of milk through heating has also been suggested as a means of differentiating between raw and heated milk.[4]

12. Saturation with Magnesium Sulphate.—Place about 5 c.c. of milk in a test-tube and saturate with solid magnesium sulphate. What is this precipitate?

13. Influence of Gastric Rennin on Milk.—Prepare a series of five tubes as follows:

(a) 5 c.c. of fresh milk + 0.2 per cent HCl (add drop by drop until a precipitate forms).

(b) 5 c.c. of fresh milk + 5 drops of rennin solution.[5]

(c) 5 c.c. of fresh milk + 10 drops of 0.5 per cent Na_2CO_3.

(d) 5 c.c. of fresh milk + 10 drops of ammonium oxalate.

(e) 5 c.c. of fresh milk + 5 drops of 0.2 per cent HCl.

[1] "Trikresol" is the trade name of an antiseptic which contains the three cresols in approximately equal proportions.

[2] Probably some organic peroxide or quinoid compound.

[3] Wilkinson and Peters: *Z. Nahr-Genussm.*, 16, No. 3, p. 172.

[4] Van Slyke and Keeler: *Jour. Biol. Chem.*, 42, 41, 1920.

[5] Any commercial rennin or rennet preparation or an extract of the gastric mucosa of the pig may be employed.

Now to each of the tubes (c), (d) and (e) add 5 drops of rennin solution. Place the whole series of five tubes at 40°C. and after 10–15 minutes note what is occurring in the different tubes. Give a reason for each particular result.

14. Preparation of Casein.[1]—Fill a large beaker one-third full of skimmed (or centrifugated) milk and dilute it with an equal volume of water. Add dilute hydrochloric acid until a flocculent precipitate forms. Stir after each acidification and do not add an excess of the acid as the precipitate would dissolve. Allow the precipitate to settle, decant the supernatant fluid, and reserve it for use in later (15–18) experiments. Filter off the precipitate of casein and remove the excess of moisture by pressing it between filter papers. Transfer the casein to a small beaker, add enough 95 per cent alcohol to cover it and stir for a few moments. Filter, and press the precipitate between filter papers to remove the alcohol. Transfer the casein again to a small dry beaker, cover the precipitate with ether and heat on a water-bath for ten minutes, stirring continuously. Filter (reserve the filtrate), and press the precipitate as dry as possible between filter papers. Open the papers and allow the ether to evaporate spontaneously. Grind the precipitate to a powder in a mortar. Upon the casein prepared in this way make the following tests:

(a) Solubility.—Try the solubility in water, sodium chloride, dilute acid and alkali.

(b) Millon's Reaction.—Make the test according to the directions given on page 162.

(c) Biuret Test.—Make the test according to directions given on page 164.

(d) Glyoxylic Acid Reaction (Hopkins-Cole).—Make the test according to the directions given on page 163.

(e) Unoxidized Sulphur.—Test for unoxidized sulphur according to the directions given on page 173. The sulphur content of casein is rather low, e.g., about 0.065 per cent.

(f) Fusion Test for Phosphorus.—Test for phosphorus by fusion according to directions given on page 194. Casein contains 0.7 per cent of phosphorus.

15. Coagulable Proteins of Milk.—Place the filtrate from the original casein precipitate in a casserole and heat, on a wire gauze, over a free flame. As the solution concentrates, a coagulum consisting of lactalbumin and lactoglobulin will form. Continue to concentrate the solution until the volume is about one-half that of the original solution. Filter off the coagulable proteins (reserve the filtrate) and test them as follows:

(a) Millon's Reaction.—Make the test according to the directions given on page 162.

(b) Biuret Test.—Make the test according to the directions given on page 164.

(c) Glyoxylic Acid Reaction (Hopkins-Cole).—Make the test according to the directions given on page 163.

16. Detection of Calcium Phosphate.—Evaporate the filtrate from the coagulable proteins, on a water-bath, until crystals begin to form. It may be necessary to concentrate to 15 c.c. before any crystallization will be observed. Cool the solution, filter off the crystals (reserve the filtrate), and test them as follows:

[1] Another method for the preparation of pure casein is described by Bosworth: *Am. Jour. Dis. Child.*, 22, 193, 1921. An excellent preparation of casein may be had from The Harris Laboratories Inc., Tuckahoe, New York.

(a) Microscopical Examination.—Examine the crystals and compare them with those in Fig. 170.

(b) Dissolve the crystals in nitric acid. Test part of the acid solution for phosphates. Render the remainder of the solution slightly alkaline with ammonia, then acidify with acetic acid and add ammonium oxalate. Examine the crystals under the microscope and compare them with those in Fig. 203, page 682.

FIG. 170.—CALCIUM PHOSPHATE.

17. Detection of Lactose.—Concentrate the filtrate from the calcium phosphate until it is of a syrup-like consistency. Allow it to stand over night and observe the formation of crystals of lactose. Make the following experiments.

(a) Microscopical Examination.—Examine the crystals and compare them with those in Fig. 169, page 537.

(b) Benedict's Test.—Try Benedict's test upon the mother liquor.

(c) Phenylhydrazine Test.—Apply the phenylhydrazine test to some of the mother liquor according to the directions given on page 72.

18. Milk Fat.—(a) Evaporate the ether filtrate from the casein (Experiment 13) and observe the fatty residue. The milk fat was carried down with the precipitate of casein and was removed when the latter was treated with ether. If centrifugated milk was used in the preparation of the caseinogen the amount of fat in the ether filtrate may be very small. To secure a larger yield of fat proceed according to directions given under (b) below.

(b) To 25 c.c. of whole milk in an evaporating dish add a little sand or filter paper and evaporate the fluid to dryness on a water-bath. Grind or break up the residue after cooling and extract with ether in a flask. Filter and remove the ether from the filtrate by evaporation. How can you identify fats in the ethereal residue?

19. Saponification of Butter.—Dissolve a small amount of butter in alcohol made strongly alkaline with potassium hydroxide. Place the alcoholic-potash solution in a casserole, add about 100 c.c. of water and boil for 10–15 minutes or until the odor of alcohol cannot be detected. Place the casserole in a hood and neutralize the solution with sulphuric acid. Note the odor of volatile fatty acids, particularly butyric acid. Under certain conditions the odor of ethyl butyrate may also be detected.

20. Detection of Preservatives.—(a) Formaldehyde.—In these tests *two* controls should be run, one with *pure milk* and one with milk to which a very small amount of formaldehyde has been added.

I. Leach's Hydrochloric Acid Test.—Mix 10 c.c. of milk and 10 c.c. of concentrated hydrochloric acid containing about 0.002 gram of ferric chloride in a small porcelain evaporating dish or casserole and gradually raise the temperature of the mixture, on a water-bath, nearly to the boiling-point, with occasional stirring. If formaldehyde is present a violet color is produced, while a brown color develops in the absence of formaldehyde. In case of doubt the mixture, after having been heated nearly to the boiling-point for about one minute, should be diluted with 50–75 c.c. of water, and the color of the diluted fluid

carefully noted, since the violet color if present will quickly disappear. Formaldehyde may be detected by this test when present in the proportion 1 : 250,000.

(*b*) *Salicylic Acid and Salicylates.*—Remont's Method.[1]—Acidify 20 c.c. of milk with sulphuric acid, shake well to break up the curd, add 25 c.c. of ether, mix thoroughly, and allow the mixture to stand. By means of a pipette remove 5 c.c. of the ethereal extract, evaporate it to dryness, boil the residue with 10 c.c. of 40 per cent alcohol, and cool the alcoholic solution. Make the volume 10 c.c., filter through a *dry* paper if necessary to remove fat, and to 5 c.c. of the filtrate, which represents 2 c.c. of milk, add 2 c.c. of a 2 per cent solution of ferric chloride. The production of a *purple* or *violet* color indicates the presence of salicylic acid.

This test may form the basis of a quantitative method by diluting the final solution to 50 c.c. and comparing this with standard solutions of salicylic acid. The colorimetric comparisons may be made in a colorimeter. (See page 358.)

(*c*) *Hydrogen Peroxide.*—Add 2–3 drops of a 2 per cent aqueous solution of para-phenylenediamine hydrochloride to 10–15 c.c. of milk. If hydrogen peroxide is present a *blue* color will be produced immediately upon shaking the mixture or after allowing it to stand for a few minutes. It is claimed that hydrogen peroxide may be detected by this test when present in the proportion 1 : 40,000.

(*d*) *Boric Acid and Borates.*—To the ash, obtained according to the directions given in Experiment 4, page 546, add 2 drops of dilute hydrochloric acid and 1 c.c. of water. Place a strip of turmeric paper in the dish and after allowing it to soak for about one minute remove it and allow it to dry in the air. The presence of boric acid is indicated by the production of a deep *red* color which changes to *green* or *blue* upon treatment with a dilute alkali. This test is supposed to show boric acid when present in the proportion 1 : 8000.

(*e*) *Benzoic Acid.*—Shake 5 c.c. of strong HCl with 50 c.c. of milk in a flask. Extract by gently shaking with successive portions of ether avoiding formation of an emulsion. Evaporate off ether. Stir residue with dilute ammonia. Pour off and evaporate to dryness on a water bath to remove free NH_3. Dissolve in 1 c.c. of water and add a few drops of ferric chloride solution. A flesh colored precipitate of ferric benzoate forms.

Quantitative Analysis of Milk

1. Collection of Human Milk for Analysis.—There are two methods of obtaining samples of breast milk for analysis.[2]

First Method.—Express all the milk from one breast and mix thoroughly. Second Method.—Draw one ounce of milk before nursing and one ounce after nursing. Mix the two samples thoroughly. The best time for obtaining samples is 9–10 o'clock in the morning.

2. Specific Gravity.—This may be determined conveniently by means of a Soxhlet, Veith, or Quevenne *lactometer*. A lactometer reading of 32° denotes a specific gravity of 1.032. The determination should be made at about 60°F. and the lactometer reading corrected by adding or subtracting 0.1° for every degree F. above or below that temperature.

[1] For other tests see Sherman's Organic Analysis, Second Edition, p. 378.
[2] Talbot: *Jour. Am. Med. Ass'n*, 73, 662, 1919.

3. Fat.—(*a*) *Babcock's Centrifugal Method.*[1]—*Principle.*—The principle of this method is the destruction of organic matter other than fat by sulphuric acid and the centrifugation of the acid solution in the special tube shown in Fig. 171 and the subsequent reading of the percentage of fat by means of the tube's graduated neck. The method is one of the most satisfactory in common use and is accurate to within 0.5 per cent.

FIG. 171.—BABCOCK TUBE

Procedure.—By means of a special narrow pipette introduce milk into the tube up to the 5 c.c. mark. Now add sufficient sulphuric acid (sp. gr. 1.83–1.834) to fill the body of the tube and rotate the tube to secure a homogeneous acid-milk solution. Fill the neck of the tube with an acid-alcohol mixture.[2] Centrifuge the tube and contents for one to two minutes and read off the percentage of fat by means of the graduated neck of the tube. If the top of the fat column is not at zero it may be brought there by the addition of water and a moment's recentrifugation.

In case very rich milk (over 5 per cent fat) is under examination, it may be diluted with an equal volume of water before examination and the fat percentage multiplied by 2. In the examination of cream it is customary to dilute the sample with four volumes of water and multiply the resultant fat value by 5.

(b) Quantitative Determination of Fat in Milk by the Meigs[3] Method with Modification and Improved Apparatus by Croll.[4]—The method as stated by Dr. Meigs is: Approximately 10 c.c. of milk is carefully weighed and transferred to an ordinary 100 c.c. glass-stoppered graduated cylinder. Twenty c.c. each of distilled water and ether (0.720) are added, the ground-glass stopper tightly inserted in the bottle, and the whole shaken vigorously for five minutes. Then the bottle is carefully unstoppered, 20 c.c. 95 per cent alcohol added, the stopper reinserted and again shaken for five minutes. The bottle is now placed on a table and the contents will separate into two distinct strata, the upper of which contains practically all the fat. This stratum is carefully removed by a small pipette and transferred to a carefully weighed glass evaporating dish. The thin ether layer remaining is washed by the addition of 5 c.c. of ether. This is removed by pipetting off. This washing is repeated four times. On each addition the

[1] A modification of this method for use with sweetened dairy products, *e.g.*, ice cream, and entailing the use of a different type of centrifuge tube has been proposed by Halverson (*Jour. Ind. and Eng. Chem.*, 5, 403, 1913). Another modification involving the use of mixtures of glacial acetic, sulphuric and nitric acids instead of sulphuric acid alone has been proposed by Francis and Morgan (*Jour. Ind. and Eng. Chem.*, 9, 861, 1917). These authors use the regulation Babcock tube, and the method is applicable to the analysis of ice cream, and evaporated, malted and dried milk.

[2] This mixture consists of equal volumes of amyl alcohol and concentrated hydrochloric acid.

[3] Original paper by Dr. Arthur V. Meigs in *Philadelphia Medical Times*, July 1, 1882.

[4] Croll: *Biochem. Bull.*, 2, 509, 1913.

sides of the bottle should carefully be washed down by the fresh ether. Finally, the pipette is rinsed with a little ether. The evaporating dish with contents is now placed on a safety water-bath and the ether evaporated. The drying is continued in a hot-air oven at a temperature below 100°C. and finally completed in a desiccator to constant weight.

Croll's modification consists of subsequent repeated extraction of the end-product of evaporation with absolute ether. The combined extracts are filtered and the small filter paper is washed repeatedly with absolute ether. The combined extracts and washings are evaporated and dried as before and then weighed.

The piece of apparatus shown in Fig. 172, below was also devised by Croll to do away with the use of the pipette.[1] On closing the top with a finger and blowing into the mouth-piece, the upper stratum is forced out into the dish. The bottle is washed by simply pouring the ether into the tube. This lessens the possibility of accidental loss.

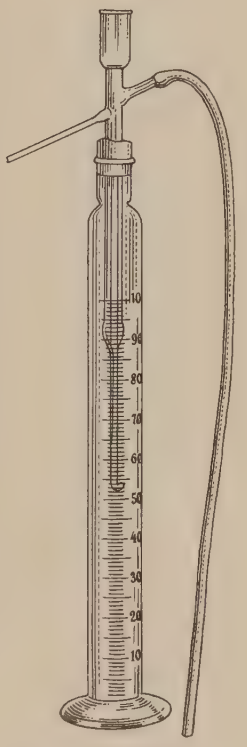


FIG. 172.—CROLL'S FAT APPARATUS.

The accuracy of the method compared with that of the Soxhlet method, using the paper-coil modification and extracting until fresh portions of absolute ether gave no further trace of extractive material, is shown by the average difference on twelve samples of human milk being only 0.017 per cent less than by the Soxhlet and on seven samples cow's milk being only 0.019 per cent less. The extreme differences in case of the human milk were—0.004 per cent and 0.044 per cent and in case of the cow's milk—0.006 per cent and —0.068 per cent.

(*c*) *Adams' Paper-coil Method.*—Introduce about 5 c.c. of milk into a small beaker, quickly ascertain the weight to centigrams, stand a fat-free coil[2] in the beaker and incline the vessel and rotate the coil in order to hasten the absorption of the milk. Immediately upon the complete absorption of the milk remove the coil and again quickly ascertain the weight of the beaker. The difference in the weights of the beaker at the two weighings represents the quantity of milk absorbed by the coil. Dry the coil carefully at a temperature below 100°C. and extract it with ether for 3–5 hours in a Soxhlet apparatus (Fig. 173). Using a safety water-bath, heat the flask containing the fat to constant weight at a temperature below 100°C.

Calculation.—Divide the weight of fat, in grams, by the weight of milk, in grams. The quotient multiplied by 100 is the *percentage of fat* contained in the milk examined.

[1] If desired a cork with two tubes may be substituted for this somewhat complicated apparatus.

[2] Very satisfactory coils are manufactured by Schleicher and Schüll.

(*d*) *Nephelometric Method of Bloor.*[1]—This method is exactly similar in principle and procedure to the method given for the determination of fat in blood. (See page 393.) One c.c. of milk is ordinarily taken.

4. Total Solids.[2]—Introduce 2–5 grams of milk into a weighed flat-bottomed platinum dish[3] and quickly ascertain the weight to milligrams. Expel the major portion of the water by heating the open dish on a water-bath and continue the heating in an air-bath or water oven at 97°–100°C. until the weight is constant. (If platinum dishes are employed this residue may be used in the determina tion of ash according to the method described below.)

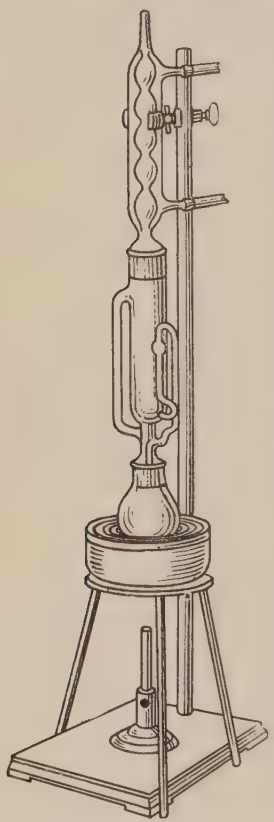


FIG. 173.—SOXHLET APPARATUS.

Calculation.[4]—Divide the weight of the residue, in grams, by the weight of milk used, in grams. The quotient is the percentage of solids contained in the milk examined.

5. Ash.—Heat the dry solids from 2–5 grams of milk, obtained according to the method just given, over a very low flame[5] until a white or light gray ash is obtained. Cool the dish in a desiccator and weigh. (This ash may be used in testing for borates according to directions on page 543.)

6. Proteins.—Introduce a known weight of milk (5–10 grams) into a 500 c.c. Kjeldahl digestion flask and add 20 c.c. of concentrated sulphuric acid and about 0.2 gram of copper sulphate. Expel the major portion of the water by heating over a low flame and finally use a full flame and allow the mixture to boil one to two hours. Complete the determination according to the directions given under Kjeldahl Method, page 711. If large amounts of milk are not available a micro method such as Koch-McMeekin (p. 717) may be used.

Calculation.—Multiply the total nitrogen content by the factor 6.37[6] to obtain the protein content of the milk examined.

[1] Bloor: *J. Am. Chem. Soc.*, 36, 1300, 1914.

[2] Shackell's method for the vacuum desiccation of frozen preparations may be used where great accuracy is desired (see *American Journal of Physiology*, 24, 325, 1909).

[3] Lead foil dishes, costing only about one dollar per gross, make a very satisfactory substitute for the platinum dishes.

[4] The percentage of total solids may be calculated from the *specific gravity* and *percentage of fat* by means of the following formula which has been proposed by Richmond:

$$S = 0.25\,L + 1.2\,F + 0.14$$

S = total solids.
L = lactometer reading.
F = fat content.

[5] Great care should be used in this ignition, the dish at no time being heated above a faint redness, as chlorides may volatilize.

[6] The usual factor employed for the calculation of protein from the nitrogen content is 6.25 and is based on the assumption that proteins contain *on the average* 16 per cent of nitrogen. This special factor of 6.37 is used to calculate the protein content from the total nitrogen, since the principal protein constituents of milk, *i.e.*, *casein* and *lactalbumin*, contain about 15.7 per cent of nitrogen.

7. Hart's Casein Method.[1]—Introduce 10.5 c.c. of milk into a 200 c.c. Erlenmeyer flask and add 75 c.c. of distilled water and 1–1.5 c.c. of 10 per cent acetic acid.[2] Mix the contents by giving the flask a vigorous rotary motion. The precipitated casein is now filtered off upon a 9–11 cm. filter paper.[3] Wash out the adsorbed and loosely combined acetic acid by means of cold water. Continue the washing of both the casein on the filter and that adhering to the flask, until the wash water has reached a volume of at least 250 c.c.

Now return the precipitate and paper to the original Erlenmeyer flask, add 75–80 c.c. of neutral (carbon dioxide-free) water, 10 c.c. of N/10 potassium hydroxide and a few drops of phenolphthalein. Stopper the flask and shake it vigorously, by hand or machine, until the casein has been brought into solution.[4] Rinse the stopper with neutral (carbon dioxide-free) water and titrate the alkaline casein solution at once with N/10 hydrochloric acid until there is a disappearance of all red color.[5]

Calculation.—Subtract the corrected[5] acid reading from the 10 c.c. of alkali used. The difference is the percentage of casein in the milk. For example, if it takes 6.7 c.c. of N/10 hydrochloric acid to titrate the alkaline solution to the end point and the check test was equivalent to 20 c.c. N/10 acid the casein value would be obtained as follows:

$$10-(6.7+0.2)=3.1 \text{ per cent casein}$$

8. Casein.—Mix about 20 grams of milk with 40 c.c. of a saturated solution of magnesium sulphate and add the salt in substance until no more will dissolve. The precipitate consists of casein admixed with a little fat and lacto-globulin. Filter off the precipitate, wash it thoroughly with a saturated solution of magnesium sulphate,[6] transfer the filter paper and precipitate to a Kjeldahl digestion flask, and determine the nitrogen content according to the directions given in a previous experiment (6).

Calculation.—Multiply the *total nitrogen* by the factor 6.37 to obtain the casein content.

9. Lactalbumin.—To the filtrate and washings from the determination of casein, in Experiment 8, add Almén's[7] reagent until no more precipitate forms. Filter off the precipitate and determine the nitrogen content according to the directions given under Proteins, page 546.

Calculation.—Multiply the *total nitrogen* by the factor 6.37 to obtain the lactalbumin content.

10. Lactose.—To about 350 c.c. of water in a beaker add 20 grams of milk, mix thoroughly, acidify the fluid with about 2 c.c. of 10 per cent acetic acid and

[1] Hart: *Jour. Biol. Chem.*, 6, 445, 1909.

[2] In general 1.5 c.c. of acetic acid gives a clear solution which filters nicely but occasionally, when the milk has a low casein value it is advisable to use less acetic acid.

[3] The process of filtration may be retarded through the packing of the casein mass upon the filter paper. In this case conduct a fine stream of cold water against the upper point of contact of filter paper and casein. By this means the casein precipitate is loosened and gathers in the apex of the filter. This procedure is very essential. It is not necessary to remove the casein which adheres to the interior of the flask.

[4] Solution is indicated by the disappearance of the white casein particles which would otherwise settle to the bottom of the flask.

[5] A check test should be run parallel with the entire determination. Even with special precautions as to neutrality, it is generally found that an acid check of 0.2–0.3 will be obtained. This check titration should be *added* to the volume of acid used in titration.

[6] Preserve the filtrate and washings for the determination of lactalbumin (Expt. 9).

[7] Almén's reagent may be prepared by dissolving 5 grams of tannic acid in 240 c.c. of 50 per cent alcohol and adding 10 c.c. of 25 per cent acetic acid

stir the acidified mixture continuously until a flocculent precipitate forms. At this point the reaction should be distinctly acid to litmus. Heat the solution to boiling for one-half hour, filter, rinse the beaker thoroughly, and wash the precipitated proteins and the adherent fat with hot water. Combine the filtrate and wash water and concentrate the mixture to about 150 c.c. Cool the solution and dilute it to 200 c.c. in a volumetric flask. Titrate this sugar solution according to directions given under Benedict's Method, page 744.

Myers[1] recommends the following procedure for the determination of lactose in milk. One part of milk is mixed with an equal volume of phosphotungstic acid solution (70.0 grams acid and 200 c.c. conc. HCl in 1 liter of water) and 2–3 parts of water. Mix well, filter until clear, and titrate the clear filtrate against Benedict's solution (25 c.c. reduced by 67 mg. of lactose).

The milk may also be clarified for the lactose determination by means of aluminum hydroxide[2] or dialyzed iron.[3] The dialyzed iron procedure is as follows: Dilute 10 gm. of milk to 25 c.c. and add about 3 c.c. of 10 per cent colloidal iron solution adding the last portion drop by drop to determine the exact amount necessary. Filter and wash with water to make the clear filtrate 100 c.c. Titrate using Benedict's method (see page 744).

The preparation of aluminum hydroxide cream and its use in protein removal are described under Nitrogen Partition, p. 712, Chapter XXXI.

Calculation.—Make the calculation according to directions given under Benedicts Method, page 744, bearing in mind that 25 c.c. of Benedicts solution is completely reduced by 0.067 gram of lactose.

11. Lactose.[4] Principle.—Lactose is determined on the protein-free filtrate of milk by a copper reduction method, following the method of Folin and Wu for blood sugar. This method is well suited to routine analyses.

Procedure.—Introduce 1.0 c.c. of milk into a 100 c.c. volumetric flask, add 2 c.c. of 10 per cent sodium tungstate. Add gradually 2 c.c. of $\frac{2}{3}$ N sulphuric acid (or 16 c.c. of N/12 acid), mix well and let stand 5 minutes. Dilute to the mark with water and filter. Into a Folin-Wu sugar tube introduce one c.c. of the filtrate and one c.c. of water. Into another tube place 2 c.c. of standard lactose solution.[5] Add 2 c.c. of the Folin-Wu alkaline copper reagent (p. 382) to each tube and heat in boiling water for 8 minutes. Cool and add 2 c.c. of phosphomolybdic acid reagent (p. 382) to each tube. Dilute to the 25 c.c. mark, mix and compare in the colorimeter.

Calculation.

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.6 \times \frac{100}{0.01} \times \frac{1}{1000} = \text{Per cent lactose.}$$

Set the unknown at the left at 20 mm., and

$$\text{Per cent lactose} = 0.3\ (\text{Reading of Standard})$$

[1] Myers: *Münch. med. Woch.*, 59, 1494, 1912.
[2] Welker and Marsh: *J. Am. Chem. Soc.*, 35, 823, 1913.
[3] Hill: *Jour. Biol. Chem.*, 20, 175, 1915.
[4] Owen and Gregg: *Jour. Lab. Clin. Med.*, 6, 220, 1920. Modified.
[5] Prepare a stock standard solution by dissolving 1.0 gm. of lactose in 0.2 per cent benzoic acid and making up to a volume of 100 c.c. The working standard is prepared by diluting 3 c.c. of the stock solution to 100 c.c. with 0.2 per cent benzoic acid, 2 c.c. = 0.6 mg. lactose.

CHAPTER XXIII

EPITHELIAL AND CONNECTIVE TISSUES
TEETH

Epithelial Tissue (Keratin).—The albuminoid *keratin* constitutes the major portion of hair, horn, hoof, feathers, nails, and the epidermal layer of the skin. There is a group of keratins the members of which possess very similar properties. The keratins as a group are insoluble in the usual protein solvents and are not acted upon by the gastric or pancreatic juices. They all respond to the xanthoproteic and Millon reactions and are characterized by containing large amounts of sulphur. Keratin from any of its sources may be prepared in a pure form by treatment, in sequence, with artificial gastric juice, artificial pancreatic juice, boiling alcohol, and boiling ether, from twenty-four to forty-eight hours being devoted to each process.

The percentage composition of some typical keratins is given in the following table:

Source		Percentage composition				
		S	N	C	H	O
Nails[1]		2.80	17.51	51.00	6.94	21.75
Horn[2]		3.20	.....	50.86	6.94	.....
Human Hair.[3]	Indian	4.82	15.40	44.06	6.53	29.19
	Japanese	4.96	14.64	42.99	5.91	31.50
	Negro	4.84	14.90	43.85	6.37	30.04
	Caucasian (adults)	5.22	15.79	44.49	6.44	28.66
	Caucasian (children)	4.93	14.58	43.23	6.46	30.80

The composition of human hair is influenced by its *color* and by the *race*, *sex*, *age* and *purity of breeding* of the individual.[3] It may be dif-

[1] Mulder: *Versuch einer allgem. physiol. Chem.*, *Braunschweig*, 1844–51.
[2] Horbaczewski: *Ladenburg's Handwörterbuch d. Chem.*, 3.
[3] Rutherford and Hawk: *Jour. Biol. Chem.*, 3, 459, 1907.

ferentiated from all other animal hair or wool by its high content of *cystine*. Human hair may yield nearly 12 per cent of this amino-acid.[1]

From the analyses of the skin of the dog, rabbit and man for mineral constituents it has been shown that there is an individual variability in composition in the same species as well as distinct differences among different species.[2]

Experiments on Epithelial Tissue

Keratin

Horn shavings or nail parings may be used in the experiments which follow:

1. **Solubility.—Test the solubility of keratin in water, dilute and concentrated acid and alkali.**
2. **Millon's Reaction.**
3. **Xanthoproteic Reaction.**
4. **Glyoxylic Acid Reaction (Hopkins-Cole).**
5. **Test for Unoxidized Sulphur.**

CONNECTIVE TISSUE

I. WHITE FIBROUS TISSUE

The principal solid constituent of white fibrous connective tissue is the albuminoid collagen. This body is also found in smaller percentage in cartilage, bone, and ligament, but the collagen from the various sources is not identical in composition. In common with the keratins, collagen is insoluble in the usual protein solvents. It differs from keratin in containing less sulphur. One of the chief characteristics of collagen is, according to Hofmeister, the property of being hydrolyzed by boiling acid or water with the formation of *gelatin*. Emmett and Gies[3] claim that under these conditions there is an intramolecular rearrangement of collagen and the resultant gelatin is consequently not the product of hydrolysis. The liberation of ammonia from the collagen during the process apparently confirms this view. Collagen gives Millon's reaction as well as the xanthoproteic and biuret tests.

The form of white fibrous tissue most satisfactory for general experiments is the *tendo Achillis* of the ox. According to Buerger and Gies[4] the fresh tissue has the following composition:

Water	62.87%
Solids	37.13
Inorganic matter	0.47
Organic matter	36.66
Fatty substance (ether-soluble)	1.04
Coagulable protein	0.22
Mucoid	1.28
Elastin	1.63
Collagen	31.59
Extractives, etc	0.90

[1] Buchtala: *Zeit. physiol. Chem.*, 85, 246, 1913.
[2] Brown: *Jour. Biol. Chem.*, 68, 729, 1926.
[3] Emmett and Gies: *Jour. Biol. Chem.*, 3, xxxiii (Proceedings), 1907.
[4] Buerger and Gies: *Am. Jour. Physiol.*, 6, 219, 1901.

The mucoid just mentioned is called *tendomucoid*[1] and is a glycoprotein. It possesses properties similar to those of other connective-tissue mucoids, *e.g.*, osseomucoid and chondromucoid.

Gelatin, the body which results from the hydrolysis of collagen (see statement of Emmett and Gies, p. 550), is sometimes classed as an albuminoid (see Chapter V). It is probably better to consider gelatin as a protein derivative not properly belonging to any of the recognized classes of proteins. It responds to many of the protein tests but differs from the keratins and collagen in being easily digested and absorbed. Gelatin is not a satisfactory substitute for the protein constituents of a normal diet, however, since a certain portion of its nitrogen is not available for the uses of the organism. Gelatin from cartilage differs from gelatin from other sources in containing a lower percentage of nitrogen. Cystine and tryptophane are not numbered among the decomposition products of gelatin, hence it gives little or no test for neutral sulphur and does not respond to the glyoxylic acid reaction. Tyrosine is absent or present in traces only so that gelatin generally fails to give Millon's reaction.

Experiments on White Fibrous Tissue

The *tendo Achillis* of the ox may be taken as a satisfactory type of the white fibrous connective tissue.

1. Preparation of Tendomucoid.—Dissect away the fascia from about the tendon and cut the clean tendon into small pieces. Wash the pieces in running water, subjecting them to pressure in order to remove as much as possible of the soluble protein and inorganic salts. This washing is very important. Transfer the washed pieces of tendon to a flask and add 300 c.c. of half-saturated lime water.[2] Shake the flask at intervals for twenty-four hours. Filter off the pieces of tendon and precipitate the mucoid with dilute hydrochloric acid. Allow the mucoid precipitate to settle, decant the supernatant fluid and filter the remainder. Test the mucoid as follows:

(a) Solubility.—Try the solubility in water, sodium chloride, dilute and concentrated acid and alkali.

(b) Biuret Test.—First dissolve the mucoid in potassium hydroxide solution and then add a dilute solution of copper sulphate.

(c) Test for Unoxidized Sulphur.

(d) Hydrolysis of Tendomucoid.—Place the remainder of the mucoid in a small beaker, add about 30 c.c. of water and 2 c.c. of dilute hydrochloric acid and boil until the solution becomes dark brown. Cool the solution, neutralize it with concentrated potassium hydroxide, and test by Benedict's test. With a reduction of Benedict's solution and a positive biuret test what do you conclude regarding the nature of tendomucoid?

2. Collagen.—This substance is present in the tendon to the extent of about 32 per cent. Therefore in making the following tests upon the pieces of

[1] Cutter and Gies: *Am. Jour. Physiol.*, 6, 155, 1901.

[2] Made by mixing equal volumes of *saturated* lime water and water from the faucet.

tendon from which the mucoid, soluble protein, and inorganic salts were removed in the last experiment, we may consider the tests as being made upon collagen.

(a) Solubility.—Cut the collagen into very fine pieces and try its solubility in water and dilute and concentrated acid and alkali.

(b) Millon's Reaction.

(c) Biuret Test.

(d) Xanthoproteic Reaction.

(e) Glyoxylic Acid Reaction (Hopkins-Cole).

(f) Test for Unoxidized Sulphur.—Take a large piece of collagen in a test-tube and add about 5 c.c. of potassium hydroxide solution. Heat until the collagen is partly decomposed, then add 1–2 drops of lead acetate and again heat to boiling.

(g) Formation of Gelatin from Collagen.—Transfer the remainder of the pieces of collagen to a casserole, fill the vessel about two-thirds full of water and boil for several hours, adding water at intervals as needed. By this means the collagen is transformed and a body known as gelatin is produced (see page 551).

3. Gelatin.—On the gelatin formed from the transformation of collagen in the above experiment (g), or on gelatin furnished by the instructor make the following tests:

(a) Solubility.—Try the solubility in the ordinary solvents (see page 71) and in hot water.

(b) Millon's Reaction.

(c) Glyoxylic Acid Reaction (Hopkins-Cole).—Conduct this test according to the modification given on page 172.

(d) Test for Unoxidized Sulphur.

Make the following tests upon a solution of gelatin in hot water:

(a) Precipitation by Mineral Acids.—Is it precipitated by strong mineral acids such as concentrated hydrochloric acid?

(b) Salting-out Experiment.—Saturate a little of the solution with solid ammonium sulphate. Is the gelatin precipitated? Repeat the experiment with sodium chloride. What is the result?

(c) Precipitation by Metallic Salts.—Is it precipitated by metallic salts such as copper sulphate, mercuric chloride, and lead acetate?

(d) Coagulation Test.—Does it coagulate upon boiling?

(e) Precipitation by Alkaloidal Reagents.—Is it precipitated by such reagents as picric acid, tannic acid, and trichloracetic acid?

(f) Biuret Test.—Does it respond to the biuret test?

(g) Precipitation by Alcohol.—Fill a test-tube one-half full of 95 per cent alcohol and pour in a small amount of concentrated gelatin solution. Do you get a precipitate? How would you prepare pure gelatin from the tendo Achillis of the ox?

II. YELLOW ELASTIC TISSUE (ELASTIN)

The *ligamentum nuchæ* of the ox may be taken as a satisfactory type of the yellow elastic connective tissue. The principal solid constituent of this tissue is *elastin*, a member of the albuminoid group.

In common with the keratins and collagen, elastin is an insoluble body and gives the protein color reactions. It differs from keratin principally in the fact that it may be digested by enzymes and that it contains a very small amount of sulphur.

It has been demonstrated that elastin has the property of adsorbing pepsin from the gastric juice and thus protecting it so the enzyme can function later in the intestine[1] (see Chapter on Gastric Digestion).

Yellow elastic tissue also contains mucoid and collagen but these are present in much smaller amount than in white fibrous tissue, as may be seen from the following percentage composition of the fresh *ligamentum nuchæ* of the ox as determined by Vandegrift and Gies.[2]

Water		57.57%
Solids		42.43
Inorganic matter	0.47	
Organic matter	41.96	
Fatty substance (ether-soluble)	1.12	
Coagulable protein	0.62	
Mucoid	0.53	
Elastin	31.67	
Collagen	7.23	
Extractives, etc.	0.80	

Experiments on Elastin

1. Preparation of Elastin (Richards and Gies).[3]—Cut the ligament into fine strips, run it through a meat chopper and wash the finely divided material in cold, running water for 24–48 hours. Add an excess of half-saturated lime water, (see note at the bottom of page 551) and allow the hashed ligament to extract for 48–72 hours. Decant the lime water, remove all traces of alkali by washing in water and then boil in water with repeated renewals until only traces of protein material can be detected in the wash water. Decant the fluid and boil the ligament in 10 per cent acetic acid for a few hours. Treat the pieces with 5 per cent hydrochloric acid at room temperature for a similar period, extract again in hot acetic acid and in cold hydrochloric acid. Wash out traces of acid by means of water and then thoroughly dehydrate by boiling alcohol and boiling ether in turn. Dry in an air-bath and grind to a powder in a mortar.

2. Solubility.—Try the solubility of the finely divided elastin, prepared by yourself or furnished by the instructor, in the ordinary solvents (see page 71). How does its solubility compare with that of collagen?

3. Millon's Reaction.

4. Xanthoproteic Reaction.

5. Biuret Test.

6. Glyoxylic Acid Reaction (Hopkins-Cole).—Conduct this test according to the modification given on page 172.

7. Test for Unoxidized Sulphur.

[1] Abderhalden and Meyer: *Zeit. physiol. Chem.*, 74, 67, 1911.
[2] Vandegrift and Gies: *Am. Jour. Physiol.*, 5, 287, 1901.
[3] Richards and Gies: *Am. Jour. Physiol.*, 7, 93, 1902.

III. CARTILAGE

The principal solid constituents of the matrix of cartilaginous tissue are *chondromucoid*, *chondroitin-sulphuric acid*, *chondroalbumoid* and *collagen*. Chondromucoid[1] differs from the mucoids isolated from other connective tissues in the large amount of chondroitin-sulphuric acid obtained upon decomposition. Besides being an important constituent of all forms of cartilage, chondroitin-sulphuric acid has been found in bone, ligament, the mucosa of the pig's stomach, the kidney of the ox, the inner coats of large arteries and in human urine. It may be decomposed through the action of acid and yields a nitrogenous body known as *chondroitin* and later this body yields *chondrosin*. Chondrosin is also a nitrogenous body and has the power of reducing Benedict's solution more strongly than dextrose. Levene and La Forge[2] claim the reducing action of chondrosin to be due to an *hexosamine isomeric with glucosamine*. Sulphuric acid is a by-product in the formation of chondroitin, and acetic acid is a by-product in the formation of chondrosin.

Chondroalbumoid is similar in some respects to elastin and keratin. It differs from keratin in being soluble in gastric juice and in containing considerably less sulphur than any member of the keratin group. It gives the usual protein color reactions.

Experiments on Cartilage

1. Preparation of the Cartilage.—Boil the trachea of an ox in water until the cartilage rings may be completely freed from the surrounding tissue. Use the cartilage so obtained in the following experiments:

2. Solubility.—Cut one of the rings into very small pieces and try the solubility of the cartilage in water and dilute and concentrated acid and alkali.

3. Millon's Reaction.

4. Xanthoproteic Reaction.

5. Glyoxylic Acid Reaction (Hopkins-Cole).—Conduct this test according to the modification given on page 172.

6. Test for Unoxidized Sulphur.

7. Preparation of Cartilage Gelatin.—Cut the remaining cartilage rings into small pieces, place them in a casserole with water and boil for several hours. Filter while the solution is still hot. Observe that the filtrate soon becomes more or less solid. What is the reason for this? Bring a portion of the material into solution by heat and try the following tests:

(a) Biuret Test.

(b) Test for Unoxidized Sulphur.

(c) To about 5 c.c. of the solution in a test-tube add a few drops of barium chloride. Do you get a precipitate, and if so to what is the precipitate due?

[1] For discussion of Mucoids etc. see Levene: *Hexosamines and Mucoproteins*, Longmans, Green & Co., 1925.

[2] Levene and La Forge: *Proc. Soc. Exp. Biol. and Med.*, 11, 124, 1914.

(d) To about 5 c.c. of the solution in a test-tube add a few drops of dilute hydrochloric acid and boil for a few moments. Now add a little barium chloride to this solution. Is the precipitate any larger than that obtained in the preceding experiment? Why?

(e) To the remainder of the solution add a little dilute hydrochloric acid and boil for a few moments. Cool the solution, neutralize with solid potassium hydroxide, and try Benedict's test. Explain the result.

IV. OSSEOUS TISSUE

Of the solids of bone about equal parts are organic and inorganic matter. The organic portion, called *ossein*, may be obtained by removing the inorganic salts through the medium of dilute acid. Ossein is practically the same body which is termed collagen in the other connective tissues, and in common with collagen yields gelatin upon being boiled with dilute mineral acid.

In common with the other connective tissues bone contains a mucoid and an albuminoid. Because of their origin these bodies are called *osseomucoid* and *osseoalbumoid*. Osseomucoid, when boiled with hydrochloric acid, yields sulphuric acid and a substance capable of reducing Benedict's solution. The composition of osseomucoid is very similar to that of tendomucoid and chondromucoid (see page 178).

The inorganic basis of the dry, fat-free bone is a chemical substance, not a mixture. This fact is indicated by the uniform composition of the bones of fasting animals as well as by the definite relationship existing between the elements present. Bones of normal and fasting animals of the same species present no profound differences in percentage composition. The percentage composition of the dry, fat-free femurs of two dogs[1] after the animals had fasted for 104 and 14 days respectively was as follows:

Dog No.	Length of fast	Ash	N	CaO	MgO	P_2O_5
1.	104 days	61.50	4.6	33.3	0.8	12.80
2.	14 days	61.65	4.1	33.1	0.9	12.90

The marked uniformity in composition notwithstanding the wide variation in the fasting periods is significant. The tensile strength of the femur of the dog has been found to be at least 25,000 pounds to the square inch[1] whereas that of oak is 10,000 and that of cast iron 20,000 pounds to the square inch.

In the case of chicken bones Wheeler[2] has reported the replacement of calcium by strontium.

[1] Johnston and Hawk: Unpublished data. For data on a 117-day fast by dog No. 1, see Howe, Mattill and Hawk: *Jour. Biol. Chem.*, 11, 103, 1912.

[2] Wheeler: Bulletin 468. New York Agric. Expt. Sta., pp. 1–43, 1919.

The *percentage* composition of *normal* human bone and of bone from a case of *osteomalacia* is given in the following table:[1]

Constituent	Kind of bone	
	Normal	Osteomalacia
Calcium (CaO)	28.85	15.44
Magnesium (MgO)	0.14	0.57
Phosphorus (P_2O_5)	19.55	12.01
Sulphur (S)	0.14	0.55

Hammett[2] has shown in the case of albino rats that there is an increase in calcium and a decrease in magnesium and phosphorus during the first 75 days of growth. Moreover the bones of the female have a higher calcium value than do the bones of the male.

Experiment on Osseous Tissue

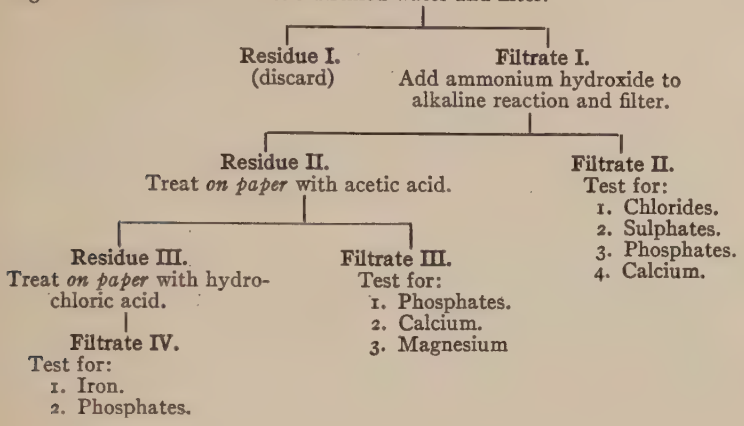
Qualitative Analysis of Bone Ash.—Take 1 gram of bone ash in a small beaker and add a little dilute nitric acid. What does the effervescence indicate? Stir thoroughly and when the major portion of the ash is dissolved add an equal volume of water and filter. To the acid filtrate add ammonium hydroxide to alkaline reaction. A heavy white precipitate of phosphates results. (What phosphates are precipitated here by the ammonia?) Filter and test the filtrate for chlorides, sulphates, phosphates, and calcium. Add dilute acetic acid to the precipitate on the paper and test a little of this filtrate for calcium and phosphates. Heat the remainder of the filtrate to boiling and add $[NH_4]_2CO_3$ and NH_4Cl slowly to this hot solution as long as a precipitate forms. Filter off the precipitate of $CaCO_3$ and wash with hot water until free from alkali.[3] To the filtrate add a solution of Na_2HPO_4, make strongly alkaline with NH_4OH, and note the formation of a white precipitate of ammonium magnesium phosphate $[NH_4MgPO_4]$. Examine the crystals under the microscope and compare with those shown in Fig. 195, page 633. To the precipitate on the filter paper, which was insoluble in acetic acid add a little dilute hydrochloric acid and test this last filtrate for phosphates and iron.

Reference to the following scheme may facilitate the analysis.

[1] McCrudden: *Jour. Biol. Chem.*, 7, 199, 1910.
[2] Hammett: *Jour. Biol. Chem.*, 64, 685, 1925; 64, 693, 1925.
[3] Magnesium is not precipitated here because of presence of NH_4Cl.

BONE ASH

Add dilute nitric acid, stir thoroughly and after the major portion of the ash has been brought into solution add a little distilled water and filter.



V. ADIPOSE TISSUE

Adipose tissue consists almost entirely of a mixture of fats. For discussion and experiments see chapter on Fats, page 245.

VI. TEETH

TEETH are composed of enamel, dentine and cement. The cement and dentine possess practically the same chemical composition as bone. The enamel is the product of an epithelial tissue. It is the hardest substance in the body and contains the smallest amount of water (3–10 per cent).[1] According to Bertz[2] the enamel and dentine of human teeth have the following percentage composition:

Dentine		Enamel
38.18	CaO	50.22
1.51	MgO	0.73
30.24	P_2O_5	40.69
29.15	Organic substance	6.82

It will be seen that *calcium phosphate* is the predominant constituent of each. Gautier has shown that enamel and apatite (crystalline phosphate rock) have nearly the same composition ($CaFCa_4(PO_4)_3$.)

In *dental caries* there may be a pronounced loss of inorganic matter and a corresponding gain in water and organic substance. These analyses by Kühns[3] illustrate this change in percentage composition:

[1] Aron: *Oppenheimer's Handbuch*, Zweiter band. 2. teil, page 208.
[2] Bertz: *Thesis*, Würzburg 1899, p. 36.
[3] Kühns: *D. Monatsschr. Zahnheilk*, 13, 361 and 450.

Sound Teeth		Dental Caries
4.27	H_2O	10.91
52.90	$Ca_3(PO_4)_2$	14.47
12.93	$CaCO_3$	7.92
1.08	$Mg_3(PO_4)_2$	0.35
28.39	Organic Substance	66.38

Proper amounts of antirachitic substance, calcium and phosphorus are essential in the diet if the teeth are to be properly calcified.[1] Absence of vitamin C in the diet leads to impairment affecting particularly the pulp of the teeth.

Analysis of Teeth.—Place a tooth in 25 c.c. of dilute nitric acid and allow to stand overnight. On the solution obtained run an analysis for inorganic elements according to the procedure given for bone ash (p. 557). Separate analyses may be made on dentine and enamel. Note the very slight amount of organic matter in enamel.

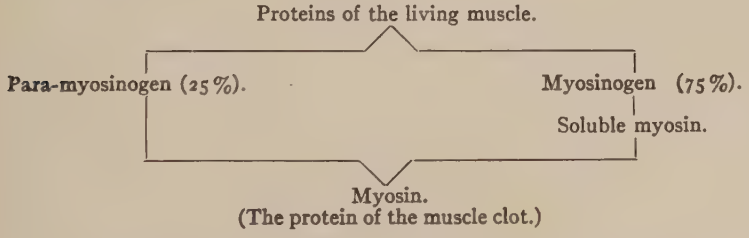
[1] Mellanby, Pattison and Proud: *Brit. Med. J.*, 2, 354, 1924.
Howe, *J. Am. Med. Assn.*, 79, 1565, 1922.
McCollum and Simmonds: The Newer Knowledge of Nutrition, Macmillan, New York, 1925.

CHAPTER XXIV

MUSCULAR TISSUE

THE muscular tissues are divided physiologically into the voluntary (striated) and the involuntary (non-striated or smooth). In the chemical examination of muscular tissue the voluntary form is generally employed. Muscle contains about 25 per cent of solid matter, of which about four-fifths is protein material and the remaining one-fifth extractives and inorganic salts.

The proteins are the most important of the constituents of muscular tissue. In the living muscle we find two proteins, *myosinogen* and *para-myosinogen*. These may be shown to be present in *muscle plasma* expressed from fresh muscles. In common with the plasma of the blood this muscle plasma has the power of coagulating, and the clot formed in this process is called *myosin*. According to Halliburton[1] and others in the onset of *rigor mortis* we have an indication of the formation of this myosin clot within the body. The relation between the proteins of *living* and *dead* muscle is represented graphically by Halliburton as follows:



Of the total protein content of living muscle about 75 per cent is made up by the *myosinogen* and the remaining 25 per cent is para-myosinogen. These proteins may be separated by subjecting the muscle plasma to fractional coagulation in the usual way. Under these conditions the para-myosinogen is found to coagulate at 47°C. and the myosinogen to coagulate at 56°C. It is also claimed by some investigators that it is possible to separate these two proteins by the fractional ammonium sulphate method, but the possibility of making

[1] Halliburton: Biochemistry of Muscle and Nerve, p. 4, 1904.

an accurate separation by this method is somewhat doubtful. It is well established that para-myosinogen is a globulin since it responds to certain of the protein precipitation tests and is insoluble in water. Myosinogen, on the contrary, is not a typical globulin since it is soluble in water. It has been called a *pseudo-globulin*. Myosin possesses the globulin characteristics. It is insoluble in water but soluble in the other protein solvents and is precipitated from its solution upon saturation with sodium chloride.

Our ideas concerning the cause of *rigor mortis* have undergone an important revision in recent years. A very attractive theory has been advanced by Meigs[1] and experimental confirmation has been accorded it by von Fürth and Lenk.[2] According to this theory, *rigor* has no connection with the coagulation of the muscle proteins and may even be hindered or prevented by such coagulation. The cause of *rigor*, from this viewpoint, lies in the *imbibition of water by the muscle colloids*. It is well known that colloids possess the property of absorbing whatever fluid may be in contact with them. Moreover, the capacity of the colloid for water is increased if the fluid is slightly acid in reaction. Therefore the postmortem production of lactic acid facilitates the imbibition of muscle fluid by the muscle colloids. Under such conditions, the fibers swell, become rigid and the condition known as *rigor mortis* results. The disappearance of rigor is believed to be due to the coagulation of the muscle protein through the agency of the accumulated lactic acid. This change is accompanied by a release of the imbibed water by the colloids, inasmuch as the capacity of a colloid for retaining fluid is lowered by coagulation.

There is a difference of opinion as to whether true *rigor* ever occurs in connection with non-striated (smooth)[3] muscle.

Under the name *extractives* we class a number of muscle constituents which occur in traces in the tissue and may be extracted by water, alcohol, or ether. There are two classes of these extractives, the *non-nitrogenous extractives* and the *nitrogenous extractives*. Grouped under the non-nitrogenous bodies we have *glycogen*, *dextrin*, *sugars*, *lactic acid*, *inositol*, $C_6H_6[OH]_6$, and *fat*. In the class of nitrogenous extractives we have *creatine*, *creatinine*, *xanthine*, *hypoxanthine*, *uric acid*, *urea*, *inosine* (*carnine*), *guanine*, *phosphocarnic acid*, *inosinic acid*, *carnosine* (*ignotine*), *taurine*, *carnitine* (*novaine*), *neosine*, *oblitine*, *carnomuscarine*, and *methylguanidine* (see formulas on pp. 192, 275 and 568). Not all of these extractives are present in the muscles of all species of animals. Other extractives besides those enumerated above have been

[1] Meigs: *American Journal of Physiology*, 26, 191, 1910.
[2] von Fürth and Lenk: *Wiener klinische Wochenschrift*, 24, 1079, 1911.
[3] Saxl: *Beiträge zur chemischen Physiologie und Pathologie*, 9, 1, 1907.

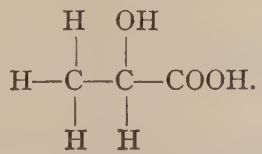
described and there are undoubtedly still others whose presence remains undetermined. A detailed consideration would, however, be unprofitable in this place.

Glycogen is an important constituent of muscle. The content of this polysaccharide in muscle varies and is markedly decreased by intense muscular activity. It is transformed into sugar and used as fuel. The liver is the organ which stores the reserve supply of glycogen and transforms it into glucose which is passed into the blood stream and so carried to the working muscle where it is synthesized into glycogen. The glycogen thus formed is then changed into glucose as the working muscle may need it.

Glycogen is a polysaccharide and has the same percentage composition as starch and dextrin. It resembles starch in forming an opalescent solution and resembles dextrin in being very soluble, in giving reddish color with iodine and in being dextro-rotatory. Glycogen may be prepared from muscle by extracting with boiling water and then precipitating the glycogen from the aqueous solution by alcohol; dilute or concentrated potassium hydroxide may also be used to extract the glycogen. Glycogen may be prepared in the form of a white, tasteless, amorphous powder. It is completely precipitated from its solution by saturation with solid ammonium sulphate, but is not precipitated by saturation with sodium chloride. It may also be precipitated by alcohol, tannic acid, or ammoniacal basic lead acetate. It has the power of holding cupric hydroxide in solution in alkaline fluids but cannot reduce it. It may be hydrolyzed with the formation of glucose by dilute mineral acids and is readily digested by amylolytic enzymes.

Mendel and Leavenworth have drawn the conclusion, from the examination of embryo pigs, that embryonic structures do not contain exceptionally large amounts of glycogen. The distribution of the glycogen was not observed to differ from that in the adult animal except that the liver of the embryo does not assume its glycogen-storing function early. They further draw the conclusion that the metabolic transformations of glycogen in the embryo and the adult are entirely analogous.

The lactic acid occurring in the muscular tissue of vertebrates is *paralactic or sarcolactic acid*,[1]



[1] This is *dextro-rotatory*, whereas *fermentation* lactic acid (*d-l*-lactic acid) is optically inactive.

The reaction of an inactive living muscle is alkaline, but upon the death of the muscle, or after the continued activity of a living muscle, the reaction becomes acid, due to the formation of lactic acid.

The lactic acid content of resting muscle is very small (not over 0.015 per cent). If a muscle be repeatedly stimulated, however, the lactic acid content may go up to 0.16 per cent. This occurs even in the absence of oxygen, as in an atmosphere of nitrogen. In this case as the lactic acid is formed there is a simultaneous and practically equivalent decrease in the glycogen of the muscle. The first of the chemical changes involved in muscular contraction are therefore anaerobic in character and involve no oxidation. If now the fatigued muscle be supplied with oxygen, an amount of oxygen will be taken up sufficient to oxidize a minor part of the lactic acid to carbon dioxide and water. The amount of oxygen taken up in this second period is equal to that which would have been taken up by the muscle if it had been supplied with oxygen throughout the period of stimulation. The major part of the lactic acid, however, is not oxidized in the recovery period but is synthesized to form glycogen again. In muscle with normal blood and oxygen supply, it is believed that eighty per cent of the lactic acid formed during a contraction is reconverted into glycogen and only twenty per cent burned. The burning of this portion is believed to furnish the energy needed for the synthetic process.

The following equations[1] summarize the changes that occur:

1. Anaerobic period.

$$\underset{\text{Glycogen}}{C_6H_{10}O_5} + H_2O = \underset{\text{Glucose}}{C_6H_{12}O_6} = \underset{\text{Lactic acid}}{2C_3H_6O_3}$$

162 gm. → 180 gm.

0.90 gm. → 1.00 gm. → 1.00 gm.

(Oxygen usage missed during anaerobic period 149 c.c.).

2. Recovery period.

1.00 gm. lactic acid

0.20 gm. oxidized.

$$\underset{0.20\text{ gm.}}{C_3H_6O_3} + \underset{149\text{ c.c.}}{3O_2} \rightarrow \underset{149\text{ c.c.}}{3CO_2} + 3H_2O$$

0.80 gm. converted to glycogen.

$$\underset{0.80\text{ gm.}}{2C_3H_6O_3} \rightarrow \underset{0.72\text{ gm.}}{C_6H_{10}O_5} + H_2O$$

The net result of these reactions is the burning of

$$0.90 - 0.72 = 0.18 \text{ gm. of glycogen.}$$

$$\underset{0.18\text{ gm.}}{C_6H_{10}O_5} + \underset{149\text{ c.c.}}{6O_2} \rightarrow \underset{149\text{ c.c.}}{6CO_2} + 5H_2O$$

[1] From Evans: "Recent Advances in Physiology," Blakiston, Philadelphia, 1926. See also Meyerhof: *Ergebnisse d. Physiol.*, 22, 328, 1923 and "*Chemical Dynamics of Life Phenomena*," Lippincott, Philadelphia, 1924. "The Mechanism of Muscular Contraction," Hill: *Physiol. Reviews*, 2, 310, 1922.

If a muscle be stimulated in the absence of oxygen lactic acid will accumulate up to a point where the acidity checks the further conversion of glycogen to lactic acid. If muscular tissue be minced the same thing is found to occur. If alkali be added to neutralize some of the acid formed the conversion is more nearly complete. The addition of phosphate with alkali may result in complete disappearance of glycogen from the tissue.

Phosphate plays a very essential role in the respiration of muscle. It plays a similar role in the fermentation of carbohydrates by yeast and its mode of action has been carefully studied.[1]

In the case of yeast fermentation it has been shown that this action cannot proceed without phosphate, and hexose diphosphate has been separated from fermenting mixtures. Hexose diphosphate has also been isolated from muscle and its formation shown to be a step preceding the formation of lactic acid. As the latter is formed the phosphate is also set free. According to Meyerhof the part played by phosphate in the production of lactic acid is as follows:

1. $5(C_6H_{10}O_5) + 5H_2O + 8K_2HPO_4 \rightarrow 4C_6H_{10}O_4(K_2PO_4)_2 + C_6H_{12}O_6 + 8H_2O \rightarrow 8C_3H_6O_3 + 8K_2HPO_4 + C_6H_{12}O_6$

For the recovery process we may have:

2. $8C_3H_6O_3 + 9K_2HPO_4 + C_6H_{12}O_6 + 6O_2 \rightarrow 4C_6H_{10}O_4\ (K_2PO_4)_2 + 6CO_2 + 14H_2O \rightarrow 4C_6H_{10}O_5 + 8K_2HPO_4 + 6CO_2 + 10H_2O$

Several theories have been proposed to account for the actual contraction of the muscle fibers but none of these has been established. Most facts support the view that it is the lactic acid formed which initiates the contraction.[2] Lactic acid solution throws voluntary muscle immersed in it into a state of rigor. The lactic acid may function by increasing osmotic pressure thus causing increased absorption of water by the contractile elements, or the acidity produced may bring about increased hydration and swelling of the colloid of these elements. Alterations in permeability of surface membranes may also occur. Relaxation probably results from diffusion of the lactic acid throughout the muscle fiber and reduction of acidity by the buffers (phosphates, etc.) of the muscle.

The lactic acid content of the blood (in the resting state about 5–20 mg. per 100 c.c.) goes up during moderate exercise to 30–60 mg. and during severe exercise to 80–200 mg. Similarly after two or three minutes of strenuous exercise the lactic acid excretion by way of the

[1] Harden: Alcoholic Fermentation; Longmans, Green, 1914. Embden and co-workers: *Zeit. Physiol. Chem.*, 113, 1, 1922: 118, 1, 1922: 127, 181, 1922: 130, 176, 1923: 141, 161 1924.

[2] Meyerhof and Lohmann: *Bioch. Zeit.*, 68, 128, 1926.

urine goes up to maxima of 86–630 mg. in the second ten minute period following and then diminishes to normal values (about 2 mg.) in from 30–50 minutes.[1] The total lactic acid excreted after one period of exercise may amount to from 140 to 1370 mg. There is also a rise in phosphate excretion after exercise; and an increased acidity of the urine. The reaction of an inactive living muscle is alkaline, but upon the death of the muscle, or after the continued activity of a living muscle, the reaction becomes acid, due to the formation of lactic acid.

Among the nitrogenous extractives of muscle, those which are of the most interest in this connection are creatine and the purine bases,

FIG. 174.—CREATINE.

xanthine and hypoxanthine. Creatine is found in varying amounts in the muscles of different species, the muscles of birds having shown the largest amount. It has also been found in the blood, the brain, in transudates and in the thyroid gland. Creatine forms colorless monoclinic prisms (Fig. 174) which are tasteless to some individuals but bitter to others[2] and which decompose with marked effervescence at about 291° (corr.) It is soluble in warm water and practically insoluble in alcohol and ether. Upon boiling a solution of creatine with dilute hydrochloric acid it is dehydrolyzed and its anhydride creatinine is formed. The theory that the creatine of ingested meat is transformed into creatinine and excreted in the urine has been proven untenable through the researches of Folin, Klercker, and Wolf and Shaffer. It is now known that under normal conditions the ingestion of creatine in no way influences the excretion of creatinine. In the case of Eck fistula dogs, however, London and Bolyarski[3] found ingested creatine to increase the output of creatinine in the urine. This finding is of impor-

[1] Liljestrand and Wilson: *Jour. Biol. Chem.*, 65, 773, 1925.
[2] Williams and Lasselle: *Jour. Am. Chem. Soc.*, 48, 536, 1926.
[3] London and Bolyarski: *Zeit. physiol. chem.*, 62, 465, 1909.

tance as throwing light upon the rôle of the liver in creatine and creatinine metabolism. In this connection it is important to note that there is no *normal* excretion of *endogenous* (see page 601) creatine, a statement proven by the fact that if no creatine be ingested none will be excreted. Folin[1] has shown that the main bulk of ingested creatine is retained in the body, unless the diet contains a large amount of protein material. In fasting the urine contains considerable creatine, *i.e.*, 120 mg. or more per day. Under certain *pathological* conditions, *e.g.*, fevers, the urine may contain *endogenous* creatine which is probably derived from the catabolism of muscular tissue, as Benedict, Mellanby, and Shaffer have suggested. Benedict and Osterberg[2] believe we may have a high creatine elimination which has no relation to the catabolism of muscle.

McCrudden[3] reports creatine in the urine in cases of infantilism, achondroplasia and cretinism the amount present being increased when the carbohydrate ingestion was increased.

It has been stated that creatine does not occur in non-striated muscle. It has, however, been found in the non-striated muscles of the *lamprey* the lowest form of vertebrates.[4]

Amberg and Morrill,[5] Sedgwick,[6] Rose[7] and Folin[8] have shown that creatine is a normal constituent of the urine of infants and children (10–15 mg. per day). Folin explains this phenomenon on the basis of the relatively high protein intake, whereas Rose believes it is due to a peculiar carbohydrate metabolism.

Besides being a normal constituent of muscle, xanthine has been found in the brain, spleen, pancreas, thymus, kidneys, testicles, liver, and in the urine. It may be obtained in crystalline form (Fig. 175, p. 566), but ordinarily it is amorphous. Xanthine is easily soluble in alkalis, less soluble in water and dilute acids, and entirely insoluble in alcohol and ether.

Hypoxanthine occurs ordinarily in those tissues and fluids which contain xanthine. It has been found, unaccompanied by xanthine, in bone marrow and in milk. Unlike xanthine it may be easily crystallized in the form of small, colorless needles. It is readily soluble in alkalis, acids, and boiling water, less soluble in cold water and practically insoluble in alcohol and ether.

[1] Folin: *Hammarsten Festschrift*, p. 15.
[2] Benedict and Osterberg: *Jour. Biol. Chem.*, 18, 195, 1914.
[3] Mc Crudden: *Jour. Expt. Med.*, 15, 457, 1912.
[4] Mellanby: *Jour. of Physiol.*, 36, 472, 1908. Wilson: *Jour. Biol Chem.*, 18, 17, 1914.
[5] Amberg and Morrill: *Jour. Biol. Chem.*, 3, 311, 1907.
[6] Sedgwick: *Jour. Am. Med. Ass'n*, 55, 1178, 1910.
[7] Rose: *Jour. Biol. Chem.*, 10, 265, 1911.
[8] Folin: *Ibid.*, 11, 253, 1912.

The predominating inorganic salt of muscle is potassium phosphate Besides this salt we have present chlorides and salts of sodium, calcium, magnesium, and iron. Sulphates are present in *traces*.

Mendel and Saiki have made some interesting observations upon the chemical composition of *non-striated* or *smooth* (involuntary) mammalian muscle, such as the urinary bladder and the muscular coat of the stomach of the pig. Hypoxanthine was found to be the predominant purine base present. Creatine and paralactic acid were also isolated. These investigators were unable to demonstrate, definitely, the presence of glycogen in the non-striated muscles studied, but state that "the tissues possess the property of transforming glycogen in the char-

FIG. 175.—XANTHINE.
After the drawings of Horbaczewski, as represented in Neubauer and Vogel. (*Ogden.*)

acteristic enzymatic way." The most important part of their investigation consists in a rather complete analysis of the inorganic constituents of these muscles. A notable difference in the relative distribution of the various inorganic constituents was observed, a difference which, according to the authors, "can be accounted for in part only by an admixture of lymph." The comparative composition of the inorganic portion of striated and non-striated muscle and of blood serum for comparison is shown in the following table:

	Per 100 parts of fresh muscle							
	K_2O	Na_2O	Fe_2O_3	CaO	MgO	Cl	P_2O_5	H_2O
Non-striated muscle (Mendel and Saiki)	0.081	0.328	0.011	0.044	0.007	0.171	0.184	80.6
Skeletal muscle (Katz)	0.306	0.210	0.008	0.011	0.047	0.048	0.487	72.9
Blood serum (Abderhalden)	0.027	0.425	.....	0.012	0.004	0.363	0.020	91.8

An interesting comparative study of the ash of the smooth muscle of the stomach of the frog and the striated muscle from the same animal has been reported by Meigs and Ryan.[1] Their data indicate "that smooth muscle contains somewhat less potassium and phosphorus and somewhat more sodium and chlorine than the striated muscle of the same animal, but that the differences in these respects between the two tissues are not by any means so marked as has sometimes been supposed." Their average figures for each type of muscle follow:

Muscle	Per 100 parts of fresh muscle									
	K	Na	Fe	Ca	Mg	P	Cl	S	Solids	H_2O
Striated	0.350	0.054	0.010	0.028	0.030	0.155	0.066	0.141	20.13	79.87
Smooth	0.325	0.073	0.0007	0.004	0.013	0.137	0.120	0.161	17.70	82.30

The preparation from which the above data for smooth muscle were obtained was shown by histological examination to consist in large part of smooth muscle fibers.

Muscular tissue is said to contain a reddish pigment called *myohematin*, which is a derivative of hemoglobin.

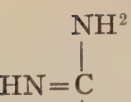
The so-called "fatigue substances" of muscle are carbon dioxide, paralactic acid, and potassium dihydrogen phosphate.

The ordinary commercial "meat extract" is composed principally of the water-soluble constituents of muscle and *contains practically nothing of nutritive value*. The protein material to which meat owes its value as an article of diet is ordinarily practically all removed in the preparation of the extract. Occasionally some preparations are found to contain proteose, which is formed from the meat proteins in the process of preparation.

Lusk[2] has shown that Liebig's extract is without influence upon the metabolism (energy) in spite of the glandular activity it is known to induce.

The structural formulas of some of the nitrogenous extractives of muscle are as follows:

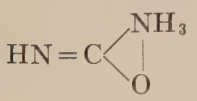
[1] Meigs and Ryan: *Journal of Biological Chemistry*, 11, 401, 1912.
[2] Lusk: *Jour. Biol. Chem.*, 13, 155, 1912.



$CH_3.N.CH_2.COOH.$

CREATINE, $C_4H_9N_3O_2$.
Methyl-guanidine acetic acid.

CREATININE, $C_4H_7N_3O$.
Creatine anhydride.



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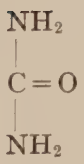
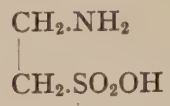
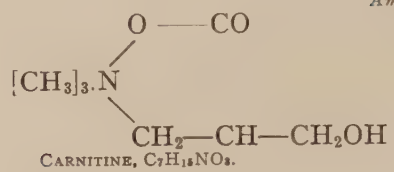


UREA, CON_2H_4.



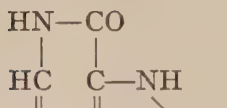
TAURINE, $C_2H_7NSO_3$.
Amino-ethyl sulphonic acid.



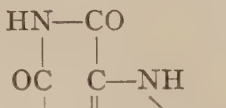
CARNITINE, $C_7H_{15}NO_3$.

Carnosine, $C_9H_{14}N_4O_3$.
Neosine, $C_6H_{17}NO_2$.
Carnic acid, $C_{10}H_{17}N_3O_5$ or $C_{10}H_{15}N_3O_5$.
Inosinic acid, $[HO]_2.PO.O.CH_2[CHOH]_3.CH:[C_5H_3N_4O]$.

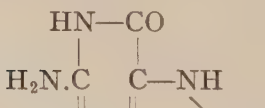
Purine Bodies.[1]—



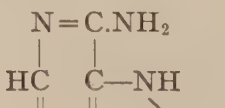
HYPOXANTHINE, $C_5H_4N_4O$.
6-oxypurine.



XANTHINE, $C_5H_4N_4O_2$.
2-6-dioxypurine.



GUANINE, $C_5H_5N_5O$.
2-amino-6-oxypurine.



ADENINE, $C_5H_5N_5$.
6-aminopurine.

EXPERIMENTS ON MUSCULAR TISSUE

I. Experiments on "Living" Muscle

1. Preparation of Muscle Plasma (Halliburton).—Wash out the blood vessels of a freshly killed rabbit with 0.9 per cent sodium chloride. This can best be

[1] For discussion of the *purine bodies* which are found as muscle extractive see Chapter VII on Nucleic Acids.

done by opening the abdomen and inserting a cannula into the aorta. Now remove the skin from the lower limbs, cut away the muscles and divide them into very small pieces by means of a meat chopper. Transfer the pieces of muscle to a mortar and grind them with clean sand and a little ice cold 5 per cent magnesium sulphate. Place in an ice-box over night. Filter off the salted muscle plasma and make the following tests:

(a) Reaction.—Test the reaction to litmus, phenolphthalein, and Congo red. What is the reaction of this fresh muscle plasma?

(b) Fractional Coagulation.—Place a little muscle plasma in a test-tube and arrange the apparatus for fractional coagulation as explained on page 169. Raise the temperature very carefully from 30°C. and note any changes which may occur and the exact temperature at which such changes take place. When the first protein (para-myosinogen) coagulates filter it off and then heat the clear filtrate as before, being careful to note the exact temperature at which the next coagulation (myosinogen) occurs. There will probably be a preliminary opalescence in each case before the real coagulation occurs. Therefore do not mistake the real coagulation-point and filter at the wrong time. What are the coagulation temperatures of these two proteins? Which protein was present in greater amount?

(c) Formation of the Myosin Clot.—Dilute a portion of the plasma with 3 or 4 times its volume of water and place it on a water-bath or in an incubator at 35°C. for several hours. A typical myosin clot should form. Note the muscle serum surrounding the clot. Now test the reaction. Has the reaction changed, and if so to what is the change due? Make a test for lactic acid. What do you conclude?

2. Preparation of Muscle Plasma (v. Fürth).—Remove the blood-free muscles of a rabbit as explained above. Finely divide by means of a meat chopper and grind in a mortar with a little clean sand and some 0.9 per cent. sodium chloride. Wrap portions of the muscle in muslin and press thoroughly by means of a tincture press or lemon squeezer. Filter and make the tests according to the directions given in the last experiment.

3. "Fuchsin-frog" Experiment.—Inject a saturated aqueous solution of Fuchsin "S" into the lymph spaces of a frog two or three times daily for one or two days, in this way thoroughly saturating the tissues with the dye. Pith the animal (insert a heavy wire or blunt needle through the occipito atlantoid membrane), remove the skin from both hind legs and expose the sciatic nerve in one of them. Insert a small wire hook through the jaws of the frog and suspend the animal from an ordinary clamp or iron ring. Pass electrodes under the exposed sciatic nerve, and after tying the other leg to prevent any muscular movement, stimulate the exposed nerve by means of make and break shocks from an induction coil. The stimulated leg responds by pronounced muscular contractions, whereas the tied leg remains inactive. Continue the stimulation until the muscles are fatigued. The muscular activity has caused the production of lactic acid and this in turn has reacted with the injected fuchsin to cause a pink or red color to develop. The muscles of the inactive leg still remain unchanged in color.

The normal color of the Fuchsin "S" when injected was red, but upon being absorbed it became colorless through the action of the alkalinity of the blood. Upon stimulating the muscles, however, as above explained, lactic acid was

formed and this acid reacted with the fuchsin and again produced the original color of the dye.

4. Production of Lactic Acid during Contraction.—Pith a frog as in the preceding experiment. Chill one hind leg with ice or a stream of CO_2. Amputate at the juncture of the femur and pelvis, remove the skin and grind muscle and bone with a little ice cold alcohol and cold sand in a cold mortar. Transfer to a beaker and allow to extract at 45°C. for 15 minutes. Filter. In the meantime the other hind leg is stimulated as in Exp. 3 and is then removed and treated in exactly the same manner as the first. Evaporate off the alcohol from both extracts on a water bath at about 80°C. Add a few c.c. of water to each, filter and again evaporate to dryness. Transfer to test tubes with the aid of 5 c.c. of concentrated sulphuric acid, add one drop of a saturated solution of copper sulphate to each and immerse in boiling water for 1–2 minutes. Cool tube in running water, add 2–3 drops of thiophene solution (10–20 drops in 100 c.c. alcohol) and replace in boiling water. Lactic acid produces a cherry red color. Compare the lactic acid reaction of the two muscles. For a more accurate quantitative method for the determination of lactic acid in muscular tissue see p. 401.

The quantities of glycogen[1] and of total and free sugars[2] also undergo change during muscular activity and may be determined quantitatively.

II. Experiments on "Dead" Muscle

1. Preparation of Myosin.—Take 25 grams of finely divided lean beef which has been carefully washed to remove blood and lymph constituents and place it in a beaker with 10 per cent sodium chloride. Stir occasionally for several hours. Strain off the meat pieces by means of cheese cloth, filter the solution and saturate it with sodium chloride in substance. Filter off the precipitate of myosin and make the tests as given below. This filtration will proceed very slowly. Myosin collects as a film on the sides of the filter paper and may be removed and tested before the entire volume of fluid has been filtered. If this precipitate remains for any length of time on the paper in contact with the air it will become transformed into the protean myosan. Test the myosin precipitate as follows:

(a) Solubility.—Try its solubility in water sodium chloride, dilute acid and alkali. Is myosin an albumin or a globulin?

(b) Xanthoproteic Reaction.—See page 163.

(c) Coagulation Test.—Suspend a little of the myosin in water in a test-tube and heat to boiling for a few minutes. Now remove the suspended material and try its solubility in 10 per cent sodium chloride. What property does this experiment show myosin to possess?

Test the filtrate from the original myosin precipitate as follows:

(a) Biuret Test.—What does this show?

(b) Place a little of the solution in a test-tube and heat to boiling. At the boiling-point add a drop of dilute acetic acid and filter. Test this filtrate for proteose with picric acid. Is any proteose present? Saturate another portion of the filtrate with ammonium sulphate and test for peptone in the usual way (see page 185). Do you find any peptone?

[1] Pflüger in Abderhalden: *Handbuch der biochemischen Arbeitsmethoden*, Berlin, 1910, Vol. 2, p. 1070.

[2] Cori and Cori: *J. Pharm. and Exp. Therap.*, 24, 465, 1924–5: *Jour. Biol. Chem.*, 63, 53, 1925.

From your experiments on "living" and "dead" muscle what are your ideas regarding the proteins of muscle?

2. Preparation of Glycogen.—Grind a few oysters or scallops[1] in a mortar with sand. Transfer to an evaporating dish, add water, and boil for 20 minutes. Note the opalescence of the solution. At the boiling-point faintly acidify with acetic acid. Why is this acid added? Filter, and divide the filtrate into two parts. Test one part of the filtrate as follows:

(a) Iodine Test.—To 5 c.c. of the solution in a test-tube add 5–10 drops of iodine solution and 2–3 drops of 10 per cent sodium chloride. What do you observe? Is this similar to the iodine test upon any other body with which we have had to deal?

If difficulty is experienced in securing a satisfactory iodine test proceed as follows: Make equal volumes of glycogen solution acid in reaction with hydrochloric acid. Boil one solution to hydrolyze the glycogen. Add equal volumes of iodine solution to each and note the more pronounced iodine reaction in the unhydrolyzed solution.

(b) Reduction Test.—Does the solution reduce Benedict's solution?

(c) Hydrolysis of Glycogen.—Add 10 drops of concentrated hydrochloric acid to 10 c.c. of the solution and boil for 10 minutes. Cool the solution, neutralize with solid potassium hydroxide and test with Benedict's solution. Does it still fail to reduce Benedict's solution? If you find a reduction how can you prove the identity of the reducing substance?

(d) Influence of Saliva.—Place 5 c.c. of the solution in a test-tube, add 5 drops of saliva and place on the bath-water at 40°C. for 10 minutes. Does this now reduce Benedict's solution?

To the second part of the glycogen filtrate add 3–4 volumes of 95 per cent alcohol. Allow the glycogen precipitate to settle, decant the supernatant fluid, and filter the remainder. Heat the glycogen on a water-bath to remove the alcohol, then subject it to the following tests:

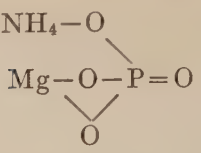
(a) Solubility.—Try its solubility in water and 10 per cent sodium chloride solution.

(b) Iodine Test.—Place a small amount of the glycogen in a depression of a test-tablet and add 2–3 drops of dilute iodine solution and a trace of a sodium chloride solution. The same wine-red color is observed as in the iodine test upon the glycogen solution.

3. Testing for Inorganic Constituents.—(a) Examination of Ash of Muscle.—Incinerate a small amount of muscular tissue, dissolve the ash in dilute hydrochloric acid. Test for potassium, phosphates, magnesium, calcium and chlorides.

(b) Demonstration of Phosphates and Magnesium in Muscle (Hürthle's Experiment).—Tease a very small piece of frog's muscle on a microscopical slide. Expose the slide to ammonia vapor for a few moments, then adjust a cover-glass, and examine the muscle fibers under the microscope. Note the large number of crystals of ammonium magnesium phosphate, distributed every-

[1] Glycogen may also be prepared from the liver of an animal which has been fed a high carbohydrate diet for 1–2 days previously. The best yield of glycogen can, however, generally be obtained from scallops. To secure best yield of glycogen the liver, scallops or oysters should be *fresh*. Canned oysters or scallops may be used if fresh ones are not available. If permitted to stand some glycogen will be converted into glucose.



where throughout the muscle fiber, thus demonstrating the abundance of phosphates and magnesium in the muscle (Fig. 195, page 633).

Separation of Extractives from Muscles

1. Creatine.—Dissolve about 10 grams of a commercial extract of meat in 200 c.c. of warm water. (Test for Protein by Biuret and Coagulation Tests, see Chapter VI.) Precipitate the inorganic constituents by neutral lead acetate, being careful not to add an excess of the reagent. Write the equations for the reactions taking place here. Allow the precipitate to settle, then filter and remove the excess of lead in the warm filtrate by hydrogen sulphide. Filter while the solution is yet warm, evaporate the clear filtrate to a syrup, and allow it to stand at least 48 hours in a cool place. Crystals of creatine should form at this point. Examine under the microscope (Fig. 174, page 564). Treat the syrup with 200 c.c. of 88 per cent alcohol, stir well with a glass rod to bring all soluble material into solution, and then filter. The purine bases have been dissolved and are in the filtrate, whereas the creatine crystals were insoluble in the 88 per cent alcohol and remain on the filter paper. Wash the crystals with 88 per cent alcohol, then remove them and bring them into solution in a little hot water. Decolorize the solution by animal charcoal and concentrate it to a small volume. Allow the solution to cool and note the separation of colorless crystals of creatine.[1]

Make the following tests on the crystals:

(a) Microscopical Examination.—Examine some crystals under the microscope and compare the form with those reproduced in Fig. 174, page 564.

(b) Transformation of Creatine into Creatinine.—Dissolve the crystals in about 30 c.c. of hot water. To one-half of the solution in a flask add an equal volume of normal hydrochloric acid and heat on a boiling water-bath for five hours with reflux condenser. The creatine has been changed into creatinine. Apply tests for creatinine as given in Chapter XXVII to the original solution as well as to the acidified solution.

Diacetyl Reaction.—To 5 c.c. of a dilute creatine solution add an equal volume of saturated sodium carbonate solution and a few drops of a solution of diacetyl. A pink color should develop. This test has been made the basis of a method for the quantitative determination of creatine.[2]

2. Hypoxanthine.—Evaporate the alcoholic filtrate from the creatine to remove the alcohol. Make the solution ammoniacal and add ammoniacal silver nitrate until precipitation ceases. The precipitate consists principally of hypoxanthine silver and xanthine silver. Collect these silver salts on a filter paper and wash them with water. Place the precipitate and paper in an evaporating dish and boil for one minute with nitric acid having a specific gravity of 1.1. Filter while hot through a double paper, wash with the same strength of nitric

[1] For an improved method of preparing pure creatine from creatinine see chapter on Physiological Constituents of the Urine.

[2] Walpole: *Jour. Physiol.*, 42, 301, 1911.

acid and allow the solution to cool. By this treatment with nitric acid hypoxanthine silver nitrate and xanthine silver nitrate have been formed. The former is insoluble in the cold solution and separates on standing. After stand-

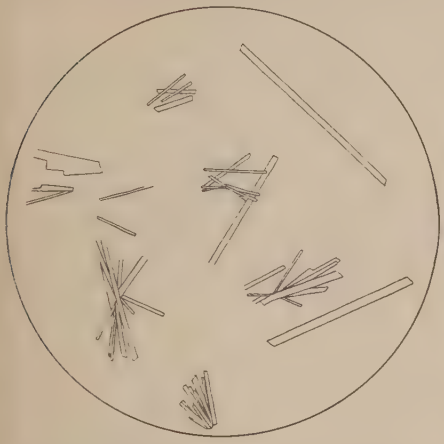


FIG. 176.—HYPOXANTHINE SILVER NITRATE. (Drawn from a student preparation by Dr. E. F. Hirsch.)

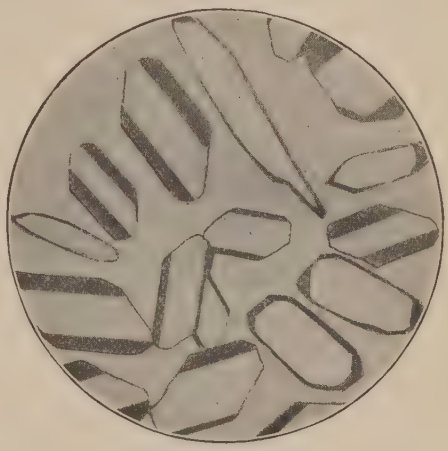


FIG. 177.—HYPOXANTHINE CHLORIDE. Hypoxanthine nitrate crystallizes in similar form. (Reproduced from crystals furnished by Professor Walter Jones.)

ing several hours filter off the hypoxanthine silver nitrate and wash with water until the wash water is only slightly acid in reaction. Examine the crystals of hypoxanthine silver nitrate under the microscope and compare them with those in

FIG. 178.—XANTHINE SILVER NITRATE.

Fig. 176. Now wash the crystals from the paper into a beaker with a little water and warm the liquid. Remove the silver by hydrogen sulphide and filter. By this means hypoxanthine nitrate has been formed and is present in the filtrate. (For crystalline form of hypoxanthine nitrate see Fig. 177, above.) Concentrate

on a water-bath to drive off hydrogen sulphide, and render the solution slightly alkaline with ammonia. Warm for a time, to remove the free ammonia, filter, concentrate the filtrate to a small volume and allow it to stand in a cool place. Hypoxanthine should crystallize in small colorless needles. Examine the crystals under the microscope.

3. Xanthine.—To the filtrate from the above experiment containing the xanthine silver nitrate add ammonia in excess. (The crystalline form of xanthine silver nitrate is shown in Fig. 178.) A brownish-red precipitate of xanthine silver forms. Filter off precipitate, suspend in water and treat with hydrogen sulphide (do not use an excess of hydrogen sulphide) then warm the mixture for a few moments and filter while hot. Concentrate the filtrate to a small volume and put away in a cool place for crystallization (Fig. 175, page 566). To obtain xanthine in crystalline form special precautions are generally necessary. Evaporate the solution to dryness and test according to directions given in Chapter VII on Nucleic Acids.

CHAPTER XXV

NERVOUS TISSUE

In common with the other solid tissues of the body, nervous tissue contains a large amount of water. The percentage of water present depends upon the particular form of nervous tissue but in all forms it is invariably greater in the gray matter than in the white. Embryonic nervous tissues also contain a larger percentage of water than the tissues of adult life. The gray matter of the brain of the fœtus, for instance, contains about 92 per cent of water, whereas the gray matter of the brain of the adult contains but 83–84 per cent of the fluid.

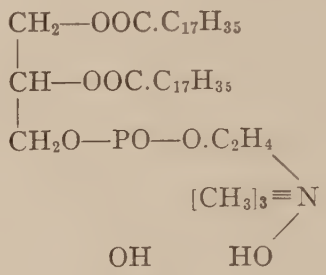
Among the solid constituents of nervous tissue are *proteins*, *cholesterol*, *cerebrosides* (cerebrin, etc.), *lecithin*, *kephalin*, *protagon* (*?*), *paranucleoprotagon*, *nuclein*, *neurokeratin*, *collagen*, *extractives*, and *inorganic salts*. The proteins are present in the greatest amount and comprise about 50 per cent of the total solids. Three distinct proteins, two globulins, and a nucleoprotein, have been isolated from the nervous tissue. The globulins coagulate at 47°C. and 70–75°C., respectively, while the nucleoprotein coagulates at 56–60°C. This nucleoprotein contains about 0.5 per cent of phosphorus (Halliburton, Levene). Nervous tissue is composed of a relatively large quantity of a variety of compounds which collectively may be grouped under the term "lipoid"—substances resembling the fats in some of their physical properties and reactions but distinct in their composition. We will class cholesterol, the cerebrosides and the phosphorized fats as lipoids.

The consideration of lipoids (or *lipins*[1]) is assuming added importance. These substances constitute one of the two great groups of *tissue colloids*, the proteins being the remaining group. So far as structure and chemical properties are concerned the various classes of lipoids are entirely unlike.

The group of *phosphorized fats* are very important constituents of nervous tissue. The best known members of this group are *lecithin*, *protagon* (*?*) and *kephalin*. Lecithin occurs in larger amount than the other members of the group, has been more thoroughly studied than the others and is apparently of greater importance. Upon decomposition lecithin yields *fatty acids*, *glycero-phosphoric acid*, and

[1] Rosenbloom and Gies: *Biochemical Bulletin*, 1, 51, 1911. The term lipoid was introduced by Overton (Studien über die Narkose, Jena, 1901, Gustav Fischer).

choline. Each lecithin molecule contains two fatty acid radicals which may be those of the same or different fatty acids. Thus we have different lecithins depending upon the particular fatty acids radicals which are present in the molecule. The formula of a typical lecithin would be the following.

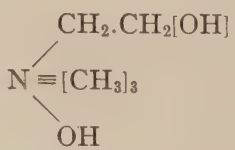


This lecithin would be called distearyl-lecithin or *cholyl-distearyl-glycero-phosphoric acid*. Upon decomposition the molecule splits according to the following reaction:

$$\underset{\text{Lecithin.}}{C_{44}H_{90}NPO_9} + 3H_2O \rightarrow \underset{\text{Stearic acid.}}{2C_{18}H_{36}O_2} + \underset{\text{Glycero-phosphoric acid.}}{C_3H_9PO_6} + \underset{\text{Choline.}}{C_5H_{15}NO_2}$$

The lecithins are not confined to the nervous tissues but are found in nearly all animal and vegetable tissues. Lecithin is a primary constituent of the cell. It is soluble in chloroform, ether, alcohol, benzene, and carbon disulphide. The chloroform or alcohol-ether solution may be precipitated by acetone. Lecithin may be caused to crystallize in the form of small plates by cooling the alcoholic solution to a low temperature. It has the power of combining with acids and bases, and the hydrochloric acid combination has the power of forming a double salt with platinic chloride.

Choline, as was indicated above, is one of the decomposition products of lecithin. It is *trimethyl-hydroxyethyl-ammonium hydroxide* and has the following formula:



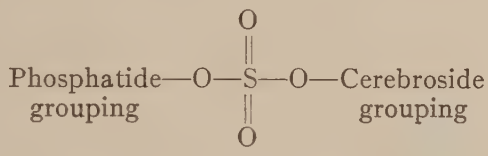
Researches have shown that great importance is to be attached to the detection of choline in the cerebro-spinal fluid and the blood in certain cases of degenerative disease of the nervous system. In this connection tests for choline (see page 580) are of interest and value.

Protagon, another nitrogenous phosphorized substance, is a body over which there has been much discussion. Upon decomposition it

is said by some investigators to yield cerebrin and the decomposition products of lecithin. It has been shown by Posner and Gies[1] as well as by Rosenheim and Tebb[2] that protagon is a mixture and has no existence as a chemical individual. Koch[3] reported data obtained from purified preparations which indicate that protagon contains at least three substances: a "phosphatide containing cholin, a cerebroside containing sugar, a complex combination of a cholin-free phosphatide with a cerebroside to which an ethereal sulphuric acid group is attached." On the basis of his data, he believed the term protagon to have no chemical significance. He proposed the term sulphatide. Koch's preparation analyzed as follows (per cent):

Choline	Sugar	Nitrogen	Phosphorus	Sulphur
1.0	12.0	2.3	1.7	1.9

He suggested the following structure:



Kephalin is the third member of the group of phosphorized fats. It is precipitated from its acetone-ether extract by alcohol. It contains about 4 per cent of phosphorus and has been given the formula $C_{42}H_{79}NPO_{13}$. Kephalin may be a stage in lecithin metabolism.

The cerebrosides are substances containing nitrogen but no phosphorus, and are important constituents of the white matter of nervous tissue. Certain ones have also been found in the spleen, pus, and in egg yolk. They may be extracted from the tissue by boiling alcohol and are insoluble in cold alcohol, cold and hot ether, and in water and dilute alkalis. The cerebroside termed cerebrin is a mixture containing phrenosin (pseudo-cerebrin or cerebron), a body yielding the carbohydrate galactose on decomposition.

Cholesterol, one of the primary cell constituents, is present in fairly large amount in nervous tissue. It occurs in two forms, *i.e.*, free and combined as an *ester*. It is claimed[4] that 99 per cent of the cholesterol of brain tissue (boy) it in the free state. It is a mon-atomic alcohol containing at least *one* double bond and possesses the formula $C_{27}H_{45}OH$ or $C_{27}H_{43}OH$. There is still some uncertainty as to the exact structure of cholesterol. It may possess a terpene structure. It

[1] Posner and Gies: *Journal of Biological Chemistry*, 1, 59, 1905–06.
[2] Rosenheim and Tebb: *Journal of Physiology*, 36 and 37, 1907–08.
[3] Koch: *Journal Biological Chemistry*, 11, March, 1912, Proceedings.
[4] Lapworth: *Jour. Path. Bact.*, 1911, p. 256.

was formerly called a "non-saponifiable fat" but since it is not changed in any way by boiling alkalis it is not a fat. It is soluble in ether, chloroform, benzene, and hot alcohol. It crystallizes in the form of thin, colorless, transparent plates (Fig. 81, page 281). Cholesterol is present in bile, and occurs abundantly in one form of biliary calculus. It is also present in blood and its quantitative determination is of clinical importance (see Chapter XVIII). It has been found in feces, wool fat, egg yolk, and milk, frequently in the form of its esters of higher fatty acids. Cholesterol is also present in the potent non-saponifiable fraction of cod liver oil although of itself it exhibits no vitamin activity. It may however be rendered antirachitic through irradiation by the ultra violet rays.[1] It is generally believed that the cholesterol present in the animal body has its origin in the vegetable kingdom. However that cholesterol may be synthesized in the animal body is shown by the fact that hens continue to lay eggs containing cholesterol when fed a cholesterol-free diet.[2] The experiments in which the tissues of the rat were found to contain the usual cholesterol content when fed a diet containing no sterols, point in the same direction.[3] However, it is probable that cholesterol is not *readily* synthesized in the body.[4]

Paranucleoprotagon is a phosphorized substance originally isolated from brain tissue by Ulpiani and Lelli and recently reinvestigated by Steel and Gies. It is said to possess lecithoprotein characteristics.

Nervous tissue yields about 1 per cent of ash which is made up in great part of alkaline phosphates and chlorides.

Experiments on the Lipoids of Nervous Tissue[5]

1. Preparation of Lecithin.[6]—Treat the finely divided brain of a sheep with ether and allow it to stand in the cold for 48–72 hours. The cold ether will extract lecithin and cholesterol. Filter and add acetone to the filtrate to precipitate the lecithin. Filter off the lecithin and test it as follows:

(a) Microscopical Examination.—Suspend a small portion in a drop of water on a slide and examine under the microscope.

[1] Hess, Weinstock and Helman: *Jour. Biol. Chem.*, 63, 305, 1925: also Rosenheim and Webster: *Lancet*, May 16, 1925; and Hess and collaborators: *Jour. Biol. Chem.*, 64, 181 and 193, 1925; 66, 145, 1925. See "Ultraviolet Radiation," Chapter XXXII.

[2] Randles and Knudson: *Jour. Biol. Chem.*, 66, 459, 1925.

[3] Channon: *Bioch. J.*, 19, 424, 1925.

[4] Gardner and Lander: *Proc. Royal Soc., London* (B), 87, 229, 1913.

[5] *Preparation of So-called Protagon.*—Divide the brain of a sheep into small pieces, treat with 85 per cent alcohol and warm on a water-bath 45°C. for two hours. Filter *hot* into a bottle or strong flask and cool to 0°C. for one-half hour by means of a freezing mixture. By this procedure both protagon and cholesterol are caused to precipitate. Filter the cold solution rapidly and treat the precipitate on the paper with ice cold ether to dissolve out the cholesterol. The protagon may now be redissolved in warm 85 per cent alcohol from which solution it will precipitate upon cooling.

[6] For the preparation of lecithin in purer form see MacLeon: *Jour. Path. Bact.*, 18, 490, 1914.

(b) Osmic Acid Test.[1]—Treat a small portion with osmic acid. What happens?

(c) Acrolein Test.—Make the acrolein test according to directions on page 250.

(d) Test for Phosphorus.—See page 194, Chapter VII.

2. Preparation of Cholesterol.—Place a small amount of finely divided brain tissue under ether and stir occasionally for one hour. Filter, evaporate the filtrate to dryness on a water-bath, and test the cholesterol according to directions given below. (If it is desired, the ether extract from the so-called protagon, or the ether-acetone filtrate from the lecithin may be used for the isolation of cholesterol. In these cases it is simply necessary to evaporate the solution to dryness on a water-bath.) Upon the cholesterol prepared by either of the above methods make the following tests:

(*a*) Microscopical Examination.—Examine the crystals under the microscope and compare them with those in Fig. 81, page 281.

(*b*) H_2SO_4 Test (Salkowski).—Dissolve a few crystals of cholesterol in a little chloroform and add an equal volume of concentrated sulphuric acid. A play of colors from bluish-red to cherry-red and purple is noted in the chloroform, while the acid assumes a marked green fluorescence.

(*c*) Acetic Anhydride-H_2SO_4 Test (Liebermann-Burchard).—Dissolve a few crystals of cholesterol in 2 c.c. of chloroform in a dry test-tube. Now add 10 drops of acetic anhydride and 1–3 drops of concentrated sulphuric acid. The solution becomes red, then blue, and finally bluish-green in color.

(*d*) *Iodine-sulphuric Acid Test.*—Place a few crystals of cholesterol in one of the depressions of a test-tablet and treat with a drop of concentrated sulphuric acid and a drop of a very dilute solution of iodine. A play of colors, consisting of violet, blue, green, and red, results.

(*e*) *Schiff's Reaction.*—To a little cholesterol in an evaporating dish add a few drops of a reagent made by adding 1 volume of 10 per cent ferric chloride to 3 volumes of concentrated sulphuric acid. Evaporate to dryness over a low flame and observe the reddish-violet residue which changes to a bluish-violet.

(*f*) Phosphorus.—Test for phosphorus according to directions given in Chapter VII, page 194. Is phosphorus present?

3. Preparation of Cerebrin.—Treat 100 grams of finely divided brain tissue, in a flask, with 200 c.c. of 95 per cent alcohol and boil on a water-bath for one-half hour, keeping the volume constant by adding fresh alcohol as needed or by the use of a reflux condenser. Filter the solution hot and stand the cloudy filtrate away for 24 hours. (If the filtrate is not cloudy concentrate it upon the water-bath until it is so.) Filter off the cerebrin (cerebrin, lecithin, kephalin, cholesterol) and test it as follows:

(a) Microscopical Examination.—Suspend a small portion in a drop of water on a slide and examine under the microscope.

(b) Solubility.—Try the solubility of cerebrin in water, 10 per cent sodium chloride and in dilute acid and alkali, and in hot and cold alcohol and hot and cold ether.

(c) Phosphorus.—Test for phosphorus according to directions in Chapter VII, page 194. How does the result compare with that on lecithin?

[1] Osmic acid serves to detect fats which contain *unsaturated fatty acid* radicals, *e.g.*, *oleic acid*, in their molecule.

(d) Place a little cerebrin on platinum foil and warm. Note the odor.

(e) Hydrolysis of Cerebrin.—Place the remaining cerebrin in a small evaporating dish, add equal volumes of water and dilute hydrochloric acid, and boil for one hour. Cool, neutralize with solid potassium hydroxide, filter, and test with Benedict's solution. Is there any reduction, and if so how do you explain it?

4. Tests for Choline.—(*a*) *Rosenheim's Periodide Test.*—Prepare an alcoholic extract of the fluid under examination, and after evaporation apply Rosenheim's iodo-potassium iodide solution[1] to a little of the residue. In a short time dark brown plates and prisms of *choline periodide* begin to form and may be detected by means of the microscope. Occasionally they are large enough to be visible to the naked eye. They somewhat resemble crystals of hemin (see page 350). If the slide be permitted to stand, thus allowing the fluid to evaporate, the crystals will disappear and leave brown oily drops. They will reappear, however, upon the addition of fresh iodine solution. v. Staněk claims that this choline compound has the formula $C_5H_{14}NOI.I_8$.

(*b*) *Rosenheim's Bismuth Test.*—Extract the fluid under examination with absolute alcohol, evaporate, and reextract the residue. Repeat the extraction several times. Dissolve the final residue in 2–3 c.c. of water and add a drop of Kraut's reagent.[2] Choline is indicated by the appearance of a bright brick-red precipitate.

[1] Prepared by dissolving 2 grams of iodine and 6 grams of potassium iodide in 100 c.c. water.

[2] Dissolve 272 grams of potassium iodide in water and add 80 grams of bismuth subnitrate dissolved in 200 grams of nitric acid (sp. gr. 1.18). Permit the potassium nitrate to crystallize out, then filter it off and make the filtrate up to 1 liter with water.

CHAPTER XXVI

URINE: GENERAL CHARACTERISTICS OF NORMAL AND PATHOLOGICAL URINE

The Secretion of Urine.—The problem of the mechanism of the secretion of urine by the kidney has occupied the attention of investigators for many years, and has produced many conflicting theories. That the renal function is a mechanical one is now well established. The filtration-reabsorption theory of Cushny[1] has been the basis of the "modern theory," and the work of other investigators, chiefly Starling and Verney[2] Wearn and Richards,[3] and Marshall and Crane[4] has led to a more satisfactory conception of the process than was hitherto known. Marshall[5] has summarized the known facts relating to the secretion of urine by the mammalian kidney in the following working hypothesis: "All of the non-colloid constituents of the plasma are

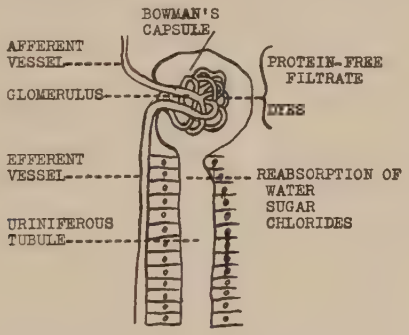


Fig. 179.—Diagram Illustrating the Secretion of Urine.

eliminated by filtration through the glomerulus; water, chloride, bicarbonate, potassium (possibly phosphate, uric acid and other bodies) are reabsorbed during the passage of the filtrate along the tubules; urea and sulphates are also absorbed to some extent under certain conditions but not as actively as chloride; ammonia, hippuric acid and possibly other bodies are formed in the renal cells and secreted, while certain foreign substances when present in the organism (phenol red) and possibly substances occurring in small amounts in the organism are secreted after a preliminary concentration in the tubule cells. The amount of glomerular filtrate on this hypothesis is considered sufficient to account for all of the sulphate and urea eliminated but not sufficient

[1] Cushny: The Secretion of Urine, Longmans, Green and Co., New York, 1926.
[2] Starling and Verney: *Proc. Roy. Soc. London*, B97, 321, 1924–25.
[3] Wearn and Richards: *Am. Jour. Physiol.*, 71, 209, 1924.
[4] Marshall and Crane: *Amer. Jour. Physiol.*, 70, 465, 1924.
[5] Marshall: *Physiol. Rev.*, 6, 440, 1926.

to account for ammonia, certain dyes (phenol red, etc.) and other bodies which are concentrated to a much greater extent than sulphate by the kidney."

By direct catheterization of the glomerular urine of frog's, and comparison of its composition with simultaneously collected bladder urine, Wearn and Richards formulated a hypothesis illustrated diagrammatically in Fig. 179. It will be noted that the chief difference from Marshall's conception is in the site of dye secretion, these workers having found small amounts of dye in the glomerular filtrate.

Volume.—The volume of urine excreted by normal individuals during any definite period fluctuates within very wide limits. The average output for twenty-four hours is placed by German writers between 1500 and 2000 c.c. This value is not strictly applicable to conditions in America, however, since it has been found that the average normal excretion of the adult male American falls within the lower values of 1000–1200 c.c. The volume-excretion is influenced greatly by the diet, particularly by the ingestion of fluids. Strenuous physical exercise causes a diminished output of urine.[1]

Certain pathological conditions cause the output of urine for any definite period to depart very decidedly from the normal output. Among the pathological conditions in which the volume of urine is *increased* above normal are the following: Diabetes mellitus, diabetes insipidus, certain diseases of the nervous system, contracted kidney, amyloid degeneration of the kidney, and in convalescence from acute diseases in general. Many drugs such as calomel, digitalis, acetates, and salicylates also increase the volume of the urine excreted. A *decrease* from the normal is observed in the following pathological conditions: Acute nephritis, diseases of the heart and lungs, fevers, diarrhœa, and vomiting.

Color.—Normal urine ordinarily possesses an amber yellow tint, the depth of the color being dependent in part upon the density of the fluid. The color of normal urine is due principally to a pigment called *urochrome;*[2] traces of *hematoporphyrin*, *urobilin*, and *uroerythrin* have also been detected. Under pathological conditions the urine is subject to pronounced variations in color and may contain many varieties of pigments. Under such circumstances the urine may vary in color from an extremely light yellow to a very dark brown or black. Vogel has constructed a color chart which is of some value for purposes of comparison. The nature and origin of the chief variations in the urinary color are set forth in tabular form by Halliburton as follows:

[1] Wilson, Long, Thompson and Thurlow: *Jour. Biol. Chem.*, 65, 755, 1925.

[2] Urochrome is believed to be identical with the yellow pigment (lactochrome) of milk *whey* (Palmer and Coolidge: *Jour. Biol. Chem.*, 17, 251, 1914).

Color	Cause of coloration	Pathological condition
Nearly colorless	Dilution, or diminution of normal pigments.	Nervous conditions: hydruria, diabetes insipidus, granular kidney.
Dark yellow to brown-red.	Increase of normal, or occurrence of pathological, pigments. Concentrated urine.	Acute febrile diseases.
Milky	Fat globules	Chyluria.
	Pus corpuscles	Purulent diseases of the urinary tract.
Orange	Excreted drugs	Santonin, crysophanic acid.
Red or reddish	Hematoporphyrin Unchanged hemoglobin	Hemorrhages, or hemoglobinuria.
	Pigments in food (logwood, madder, bilberries, fuchsin).	
Brown to brown black	Hematin	Small hemorrhages.
	Methemoglobin	Methemoglobinuria.
	Melanin	Melanotic sarcoma.
	Hydrochinol and catechol	Carbolic-acid poisoning.
Greenish yellow, greenish brown, approaching black.	Bile-pigments	Jaundice.
Dirty green[1] or blue	A dark blue scum on surface, with a blue deposit, due to an excess of indigo-forming substances.	Cholera, typhus; seen especially when the urine is putrefying.
Brown-yellow to red-brown, becoming blood-red upon adding alkalis.	Substances contained in senna, rhubarb and chelidonium which are introduced into the system.	

Transparency.—Normal urine is ordinarily perfectly clear and transparent when voided. On standing for a variable time, however, a cloud (nubecula) consisting principally of nucleoprotein or mucoid (see page 620) and epithelial cells forms. A turbidity due to the precipitation of phosphates is normally noted in urine passed after a hearty

[1] This dirty green or blue color also occurs after the use of methylene blue in the organism.

meal. The urine obtained 2–3 hours after a meal or later is ordinarily free from turbidity. Permanently turbid urines ordinarily arise from pathological conditions.

Odor.—The odor of normal urine is of a faint, aromatic type. The bodies to which this odor is due are not well known, but it is claimed by some investigators to be due, at least in part, to the presence of minute amounts of certain volatile organic acids. Dehn and Hartman[1] have recently succeeded in isolating from urine a neutral ill-smelling substance which they call *urinod*. Its empirical formula is C_6H_8O. Urinod occurs in urine to the extent of only 1–2 parts in 100,000 parts of urine. When the urine undergoes decomposition, *e.g.*, in alkaline fermentation, a very unpleasant ammoniacal odor is evolved. All urines are subject to such decomposition if allowed to stand for a sufficiently long time. Under normal conditions the urine very often possesses a peculiar odor due to the ingestion of some certain drug or vegetable. For instance, cubebs, copaiba, myrtol, saffron, tolu, and turpentine each imparts a somewhat specific odor to the urine. After the ingestion of asparagus, the urine also possesses a typical odor due to the presence of *methyl mercaptan* ($CH_3 \cdot SH$) which is formed in the intestine and eliminated in the urine.

Frequency of Urination.—The frequency of urination varies greatly in different individuals, but in general is dependent upon the amount of fluid in the bladder. In pathological conditions an inflammatory affection of the urinary tract or any disturbance of the innervation of the bladder will influence the frequency. Affections of the spinal cord which lead to an increased irritability of the bladder or a weakening of the sphincter, or any condition lowering the residual capacity of the bladder, will result in increasing the frequency of urination.

Reaction.—The mixed 24-hour urinary excretion of a normal individual ordinarily possesses an acid reaction to litmus. The actual hydrogen ion concentration varies over a wide range (pH 5.5 to 8.0), the mean being about pH 6. The reaction of the urine represents an equilibrium between a large number of acid and basic constituents, both organic and inorganic, which it contains. Although organic acids and bases play a part in producing the normal reaction, this reaction is probably, in the main, dependent upon the relative amounts of the mono- and dibasic sodium and potassium phosphates present. The monobasic sodium phosphate (NaH_2PO_4) is acid in reaction, while the dibasic phosphate (Na_2HPO_4) is alkaline in reaction. The excretion of acid or alkaline phosphate by the kidneys is one of the factors in the regulation of the neutrality of the blood and of the organism in general. The acid-

[1] Dehn and Hartman: *Jour. Am. Chem. Soc.*, 36, 2136, 1914.

ity of the urine as determined by titration runs in general parallel with the hydrogen ion concentration and seems to be dependent upon the same factors, and in more acid urines mainly on the phosphate content. Van Slyke and Palmer[1] have shown that normal men excrete organic acids equivalent to only about 6 c.c. of 0.1 N acid in twenty-four hours. Strenuous physical exercise produces an increase in hydrogen ion concentration and in acid and ammonia output.[2] (For further discussion of acidity see Chapter I.)

The mean acidity in cardio-renal diseases is high—about pH5.3 as compared with pH 6, the normal mean. In general the acidity tends to be increased in the greater number of pathological disorders.

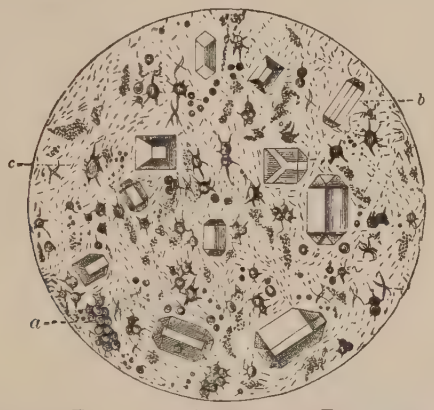


FIG. 180.—DEPOSIT IN AMMONIACAL FERMENTATION.
a, Acid ammonium urate; *b*, ammonium magnesium phosphate; *c*, bacteria.

The composition of the food is perhaps the most important factor in determining the reaction of the urine (see Chapter XXXII on Metabolism for influence of base-forming and acid-forming foods). The reaction ordinarily varies considerably according to the time of day the urine is passed.[3] For instance, for a variable length of time after a meal the urine may be neutral or even alkaline in reaction to litmus, owing to the claim of the gastric juice upon the acidic radicals to further the formation of hydrochloric acid for use in carrying out the digestive secretory function. This hypothesis has been verified experimentally.[4] This change in reaction is known as the *alkaline tide* and is common to perfectly healthy individuals. The urine may also become temporarily alkaline in reaction to litmus, as the result of ingesting alkaline carbonates or certain salts of tartaric and citric acids which may be transformed into carbonates within the organism. Normal urine upon standing for some time becomes alkaline in reaction to litmus, owing

[1] Van Slyke and Palmer: *Jour. Biol. Chem.*, 41, 567, 1920.
[2] Wilson, Long, Thompson and Thurlow: *Jour. Biol. Chem.*, 65, 755, 1925.
[3] Simpson: *Jour. Biol. Chem.*, 67, 505, 1926.
[4] Hubbard and Munford: *Proc. Soc. Exp. Biol. and Med.*, **19**, 429, 1922; Dodds and McIntosh: *Jour. Physiol.*, 57, 139, 1923.

to the inception of alkaline or ammoniacal fermentation through the agency of micro-organisms. This fermentation has no especial diagnostic value except in cases where the urine has undergone this change *within the organism* and is voided in the decomposed state. Ammoniacal fermentation is ordinarily due to cystitis or occurs as the result of infection in the process of catheterization. A microscopical examination of such urine (Fig. 180) shows the presence of *ammonium magnesium phosphate* crystals, *amorphous phosphates*, and not infrequently *ammonium urate*.

Ingestion of *acid* fruits (oranges, lemons, peaches, etc.) causes the formation of *alkaline* urine. This is due to the fact that the ash of such fruits is alkaline and when the fruits are combusted in the body *carbonates are formed*. On the other hand, bread, cereals, meats, etc., yield an *acid ash* and an acid urine.

Occasionally a urine which possesses a normal acidity when voided, upon standing instead of undergoing ammoniacal fermentation as above described, will become more strongly acid in reaction. Such a phenomenon is termed *acid fermentation*. Accompanying this increased acidity there is ordinarily a deepening of the tint of the urinary color.

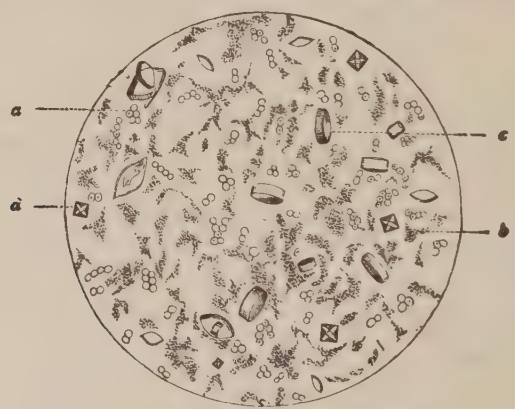


FIG. 181.—DEPOSIT IN ACID FERMENTATION.
a, Fungus; *b*, amorphous sodium urate; *c*, uric acid; *d*, calcium oxalate.

Such urines may contain *acid urates*, *uric acid*, *fungi*, and *calcium oxalate* (Fig. 181, above). On standing for a sufficiently long time any urine which exhibits acid fermentation will ultimately change in reaction, due to the inception of alkaline fermentation, and will show the microscopical deposits characteristic of such a urine.

Specific Gravity.—The specific gravity of the urine of normal individuals varies ordinarily between 1.015 and 1.025. This value is subject to wide fluctuations under various conditions. For instance, following copious water- or beer-drinking the specific gravity may fall to 1.003 or lower, whereas in cases of excessive perspiration it may rise

as high as 1.040 or even higher. Where a very accurate determination of the specific gravity is desired, use is commonly made of the *pyknometer* or of the *Westphal hydrostatic balance*. These instruments, however, are not suited for clinical use. The clinical method of determining the specific gravity is by means of a *urinometer* (Fig. 182). This affords a very rapid method and at the same time is sufficiently accurate for clinical purposes. The urinometer is always calibrated for use at a specific temperature and the observations made at any other temperature must be subjected to a certain correction to obtain the true specific gravity. In making this correction *one unit of the last order is added* to the observed specific gravity for every three degrees *above* the normal temperature and *subtracted* for every three degrees *below* the normal temperature. For instance, if in using a urinometer calibrated for 15°C. the specific gravity of a urine having a temperature of 21°C. is determined as 1.018 it is necessary to add to the observed specific gravity two units of the third order to obtain the real specific gravity of the urine. Therefore the true specific gravity, at 15°C., of a urine having a specific gravity of 1.018 at 21°C. is 1.018 + 0.002 = 1.020.

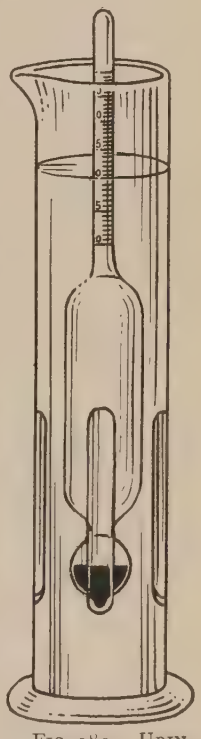


FIG. 182.—URINOMETER AND CYLINDER.

Pathologically, the specific gravity may be subjected to very wide variations. This is especially true in diseases of the kidneys. In acute nephritis ordinarily the urine is concentrated and of a high specific gravity, whereas in chronic nephritis the reverse conditions are more apt to prevail. In fact, under most conditions, whether physiological or pathological, the specific gravity of the urine is inversely proportional to the volume excreted. This is not true of diabetes mellitus, however, where the volume of urine is large and the specific gravity is also high, owing to the sugar contained in the urine.

The amount of solids eliminated in the excretion for twenty-four hours may be roughly calculated by means of *Long's coefficient, i.e.*, 2.6. The solid content of 1000 c.c. of urine is obtained by multiplying the last two figures of the specific gravity observed at 25°C. by 2.6. To determine the amount of solids excreted in twenty-four hours if the volume was 1120 c.c. and the specific gravity was 1.018 the calculation would be as follows:

(*a*) $18 \times 2.6 = 46.8$ grams of solid matter in 1000 c.c. of urine.

(*b*) $\frac{46.8 \times 1120}{1000} = 52.4$ grams of solid matter in 1120 c.c. of urine.

The coefficient of Häser (2.33) which has been in use for years probably gives values that are inaccurate for conditions existing in America. This coefficient was calculated on the basis of the specific gravity determined at a temperature of 15°C.

Freezing-point (Cryoscopy).—The freezing-point of a solution depends upon the total number of molecules of solid matter dissolved in it. The determination of the osmotic pressure by this method has come to be of some clinical importance, particularly as an aid in the diagnosis of kidney disorders. In this connection it is best to collect the urine from each kidney separately and determine the freezing-point in the individual samples so collected. By this means considerable aid in the diagnosis of renal diseases may be secured. The fluids most frequently examined cryoscopically are the blood (see page 331) and the urine. The freezing-point is denoted by Δ. The value of Δ for normal urine varies ordinarily between −1.3° and −2.3°C., the freezing-point of pure water being taken as 0°. Δ is subject to very wide fluctuations under unusual conditions. For instance, following copious water- or beer-drinking Δ may have as high a value as −0.2°C., whereas on a diet containing much salt and deficient in fluids the value of Δ may be lowered to −3°C. or even lower. The freezing-point of normal blood is generally about −0.56°C. and is not subject to the wide variations noted in the urine, because of the tendency of the organism to maintain the normal osmotic pressure of the blood under all conditions. Variations between −0.51° and −0.62°C. may be due entirely to dietary conditions, but if any marked variation is noted it can, in most cases, be traced to a disordered kidney function.

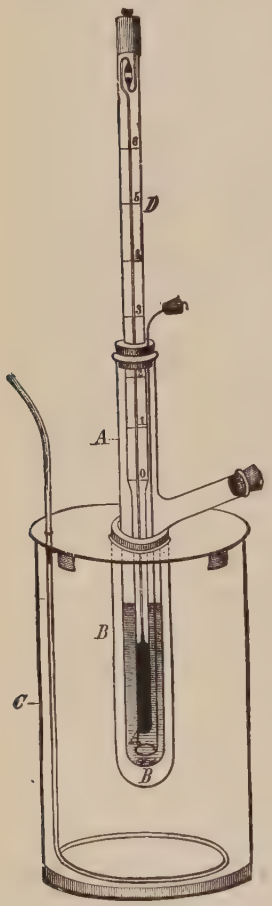


Fig. 183.—Beckmann-Heidenhain Freezing-point Apparatus. (*Long.*) *D*, a delicate thermometer; *C*, the containing jar; *B*, the outside or air mantle tube; *A*, the tube in which the mixture to be observed is placed. Two stirrers are shown, one for the cooling mixture in the jar and one for the experimental mixture.

Freezing-point determinations may be made by means of the Beckmann-Heidenhain apparatus (Fig. 183) or the Zikel pektoscope. The Beckmann-Heidenhain apparatus consists of the following parts: A strong battery jar or beaker (C) furnished

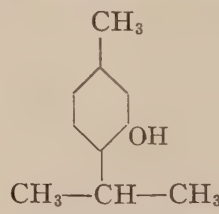
with a metal cover which is provided with a circular hole in its center. This strong glass vessel serves to hold the freezing mixture by means of which the temperature of the fluid under examination is lowered. A large glass tube (B) designed as an air-jacket, and formed after the manner of a test-tube is introduced through the central aperture in the metal cover and into this air-jacket is lowered a smaller tube (A) containing the fluid to be tested. A very delicate thermometer (D), graduated in hundredths of a degree is introduced into the inner tube and is held in place by means of a cork so that the mercury bulb is immersed in the fluid under examination but does not come into contact with any glass surface. A small platinum wire stirrer serves to keep the fluid under examination well mixed while a larger stirrer is used to manipulate the freezing mixture. (Rock salt and ice in the proportion 1:3 form a very satisfactory freezing mixture.)

In making a determination of the freezing-point of a fluid by means of the Beckmann-Heidenhain apparatus proceed as follows: Place the freezing mixture in the battery jar and add water (if necessary) to secure a temperature not lower than $-3°C$. Introduce the fluid to be tested into tube A, place the thermometer and platinum wire stirrer in position, and insert the tube into the air-jacket which has previously been inserted through the metal cover of the battery jar. Manipulate the two stirrers in order to insure an equalization of temperature and observe the course of the mercury column of the thermometer very carefully. The mercury will *gradually fall* and this gradual lowering of the temperature will be followed by a *sudden rise*. The point at which the mercury rests *after this sudden rise* is the *freezing-point*. This rise is due to the fact that previous to freezing, a fluid is always more or less *over-cooled* and the thermometer temporarily registers a temperature somewhat *below the freezing-point*. As the fluid freezes, however, there is a very *sudden* change in the temperature of the liquid and this change is imparted to the thermometer and causes the rise as indicated. It occasionally occurs that the fluid under examination is very much over-cooled and *does not freeze*. Under such circumstances a small piece of ice is introduced into it by means of the side tube noted in the figure. This so-called "inoculation" causes the fluid to freeze instantaneously. (For details of the method of determining the freezing-point consult standard works on physical or organic chemistry.)

Electrical Conductivity.—The electrical conductivity of the urine is dependent upon the number of *inorganic* molecules or ions present, and in this differs from the freezing-point which is dependent upon the total number of molecules both *inorganic* and *organic* which are in solution. The conductivity of the urine has been investigated but

slightly, but from the data secured it seems that the value generally falls below $\kappa = 0.03$. The conductivity of blood serum has been determined as $\kappa = 0.012$. Up to the present time the determination of the electrical conductivity of any of the fluids of the body has been put to very slight clinical use. Experience may show the conductivity value to be a more important aid to diagnosis than it is now considered, particularly if it is taken in connection with the determination of the freezing-point. By a combination of these two methods the portion of the osmotic pressure due respectively to electrolytes and non-electrolytes may be determined. For a discussion of electrical conductivity and the method by which it is determined (see Chapter I).

Collection and Preservation of the Urine Sample.—If any dependable data are desired regarding the *quantitative* composition of the urine the examination of the mixed excretion for twenty-four hours is *absolutely necessary*. In collecting the urine the bladder may be emptied at a given hour, say 8 A. M., the urine discarded and all the urine from that hour up to and including that passed the next day at 8 A. M., saved, thoroughly mixed, and a sample taken for analysis. Until recently it was believed that powdered thymol (*para-isopropylmetacresol*)



was a very satisfactory preservative since the excess might be removed by filtration, if desired, and it was believed that the small amount which went into solution would have no appreciable influence upon the determination of any of the urinary constituents. It appears however that thymol is not such a satisfactory urinary preservative as was believed. Evidence has been presented showing it to be unsatisfactory for the preservation of urines which contain sugar, acetone or diacetic acid, and in which it is desired to estimate the quantitative content of these constituents. Claim has also been made that thymol is not a satisfactory preservative for urines that are to be examined quantitatively for phosphates or magnesium. Thymol being a phenol will cause an inaccuracy when phenols are being determined quantitatively. Urines preserved by thymol will also give a confusing white ring when subjected to the nitric acid test for albumin (see Chapter XXVIII).

Toluene is a very satisfactory preservative for urine. In using this preservative simply overlay the urine with the toluene. Rosenbloom[1]

[1] Rosenbloom: *New York Medical Journal*, 99, 735, 1914.

claims that camphor is a very satisfactory urine preservative which does not interfere with the tests for important urinary constituents.

According to Behre and Muhlberg[1] the most satisfactory preservative consists of a 3: 2 mixture of hexamethylene-tetramine (urotropin) and salicylic acid which is used in the proportion of 50 mg. per 10 c.c. of urine. These substances produce formaldehyde in solution and it is claimed do not interfere with any of the usual tests.

In certain pathological conditions it is desirable to collect the urine passed during the *day* separately from that passed during the *night*. When this is done the urine voided between 8 A. M. and 8 P. M. may be taken as the *day sample* and that voided between 8 P. M. and 8. A. M. as the *night sample*.

The *qualitative* testing of urine samples collected at *random*, except in a few specific instances, is of no particular value so far as giving us any accurate knowledge as to the exact urinary characteristics of the individual concerned. In the great majority of cases the qualitative as well as the quantitative tests should be made upon the mixed excretion for a twenty-four-hour period as well as upon a *night sample* as above described.

[1] Behre and Muhlberg: *Jour. Lab. Clin. Med.*, 11, 785, 1926.

CHAPTER XXVII

URINE: PHYSIOLOGICAL CONSTITUENTS[1]

1. Organic Physiological Constituents

Urea.
Uric acid.
Creatinine.
Creatine.[2]

Ethereal sulphuric acids.....
- Indoxyl-sulphuric acid.
- Phenol- and *p*-cresol-sulphuric acids.
- Pyrocatechol-sulphuric acid.
- Skatoxyl-sulphuric acid.

Hippuric acid.
Oxalic acid.

Neutral sulphur compounds....
- Cystine.
- Chondroitin-sulphuric acid.
- Thiocyanates.
- Taurine derivatives.
- Oxyproteic acid.
- Alloxyproteic acid.
- Uroferric acid.

Allantoin.

Aromatic oxyacids.....
- Para-oxyphenyl-acetic acid.
- Para-oxyphenyl-propionic acid.
- Homogentisic acid.
- Uroleucic acid.
- Oxymandelic acid.
- Kynurenic acid.

Amino-acids.
Peptides.
Benzoic acid.
Nucleoprotein.
Oxaluric acid.
Glucose.

[1] It is impossible to make any *absolute* classification of the physiological and pathological constituents of the urine. A substance may be present in the urine in small amount physiologically and be sufficiently increased under certain conditions as to be termed a pathological constituent. Therefore it depends, in some instances upon the *quantity* of a constituent present whether it may be correctly termed a physiological or a pathological constituent.

[2] Normal constituent of urine of adults but found in larger amount in urine of growing children (see p. 564).

Enzymes	Pepsin.
	Gastric rennin.
	Amylase.
Volatile fatty acids	Acetic acid.
	Butyric acid.
	Formic acid.
Paralactic acid.	
Phenaceturic acid.	
Urocanic acid.	
Phosphorized compounds	Glycerophosphoric acid.
	Phosphocarnic acid.
Pigments	Urochrome.
	Urobilin.
	Uroerythrin.
Purine Bases	Adenine.
	Guanine.
	Xanthine.
	Epiguanine.
	Episarkine.
	Hypoxanthine.
	Paraxanthine.
	Heteroxanthine.
	1-Methylxanthine.

2. Inorganic Physiological Constituents

Ammonia.
Sulphates.
Chlorides.
Phosphates.
Sodium and potassium.
Calcium and magnesium.
Carbonates.
Iron.
Fluorides.
Nitrates.
Silicates.
Hydrogen peroxide.

Normal urine varies widely in composition, being influenced by *diet* and other factors. The following table represents the composition of a normal urine.[1]

[1] Vierordt: Daten und Tabellen. Jena, 1906, p. 330.

COMPOSITION OF A NORMAL URINE[1]

Volume (24 hours) 1500 c.c.

Constituent	Absolute weight, grams	Approximate percentage
Water	1440.00	96.0
Solids	60.0	4.0
Urea	35.0	2.33
Uric acid	0.75	0.05
Hippuric acid	0.7	0.05
Oxalic acid	0.015	0.001
Aromatic oxyacids	0.06	0.004
Creatinine	1.0	0.07
Thiocyanic acid (as KSCN)	0.15	0.01
Indican	0.01	0.001
Ammonia	0.65	0.04
Sodium chloride	16.5	1.1
Phosphoric acid	2.5	0.15
Total sulphuric acid[2]	2.5	0.15
Silicic acid	0.45	0.03
Potassium (K_2O)	2.5	0.15
Sodium (Na_2O)	5.0	0.3
Calcium (CaO)	0.25	0.015
Magnesium (MgO)	0.30	0.02
Iron	0.005	0.0004

[1] The values in this table were obtained from the analysis of a *single specimen* of urine and are not to be confused with *normal averages* which are based on the analysis of many normal urines. Vierordt: Daten and Tabellen, Jena, 1906, p. 330.

[2] For data as to "partition" of sulphur and nitrogen, see Chapter XXXII.

UREA, $HN=C\begin{matrix}NH_3\\|\\O\end{matrix}$ or $\begin{matrix}NH_2\\|\\C=O.\\|\\NH_2\end{matrix}$

Urea[1] is the principal end-product of the metabolism of protein substances. It was formerly believed that about 90 per cent of the total nitrogen of the urine was present as urea. Folin, however, has shown that the distribution of the nitrogen of the urine among urea and the other nitrogen-containing bodies present depends entirely upon the absolute amount of the total nitrogen excreted. He found that a decrease in the total nitrogen excretion was always accompanied by a decrease in the percentage of the total nitrogen excreted as urea, and that after so regulating the diet of a normal person as to

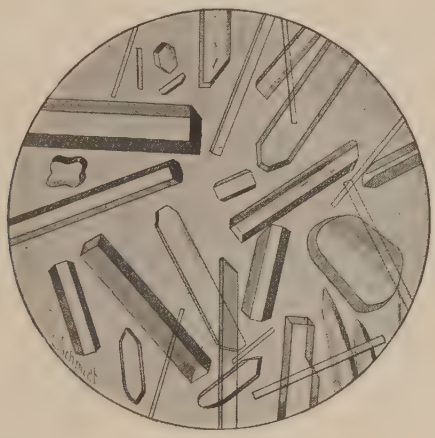


FIG. 184.—UREA.

cause the excretion of total nitrogen to be reduced to 3–4 grams in 24 hours, *only about* 60 *per cent of this nitrogen appeared in the urine as urea.* His experiments also seem to show urea to be the only one of the nitrogenous excretions which is relatively as well as absolutely decreased as a result of decreasing the amount of protein metabolized. This same investigator reports a hospital case in which only 14.7 per cent of the total nitrogen was present as urea and about 40 per cent was present as ammonia. Mörner had previously reported a case in which but 4.4 per cent of the total nitrogen of the urine was present as urea, and 26.7 per cent was present as ammonia.

[1] For review see Fearon "Biochemistry of Urea," *Physiol. Reviews* 6, 399, 1926.

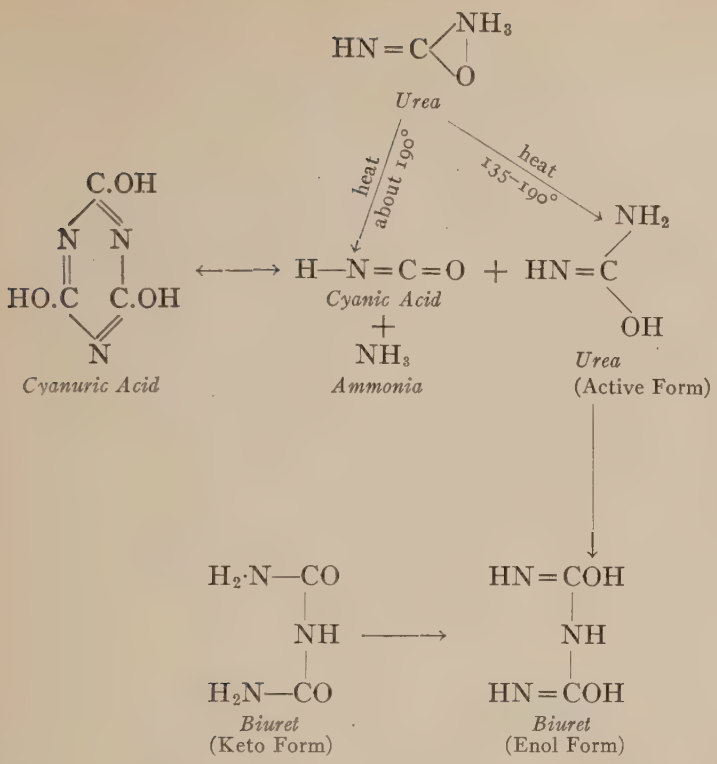
Urea occurs most abundantly in the urine of man and carnivora and in somewhat smaller amount in the urine of herbivora; the urine of fishes, amphibians, and certain birds also contains a small amount of the substance. Urea is also found in nearly all the fluids and in many of the tissues and organs of mammals. The amount excreted, under normal conditions, by an adult man in 24 hours is about 30 grams. The excretion is greatest in amount after a diet of meat, and least in amount after a diet consisting of non-nitrogenous foods; this is due to the fact that the urea output is regulated by the protein ingestion. It is true also that a non-nitrogenous diet has a tendency to decrease the metabolism of the tissue proteins and thus cause the output of urea under these conditions to fall below the output of urea observed during starvation. The output of urea is also increased after copious water- or beer-drinking. The increase is probably due *primarily* to the washing out of the tissues of the urea previously formed, but which had not been removed in the normal processes, and *secondarily* to a stimulation of protein catabolism.

Urea may be formed in the organism from amino-acids such as leucine, glycocoll, and aspartic acid: it may also be formed from ammonium carbonate $(NH_4)_2CO_3$ or ammonium carbamate, $H_4N.O.CO.NH_2$.

There are differences of opinion regarding the transformation of the substances just named into urea, but there is rather conclusive evidence that the bulk of the urea is formed in the liver; it may be formed in other organs or tissues as well. The observations of Mann and associates[1] and of Morgulis[2] have served to indicate very definitely the important role played by the liver in urea formation. The former observers claim an absolute cessation of urea formation after extirpation of the liver.

Urea crystallizes in long, colorless, four- or six-sided, anhydrous, rhombic prisms (Fig. 184), which melt at 132°C. and are soluble in water or alcohol and insoluble in ether or chloroform. If a crystal of urea is heated in a test-tube, it melts and decomposes with the liberation of ammonia and the formation of biuret and cyanuric acid. According to the Werner hypothesis the changes are as follows:

[1] Bollman, Mann and Magath: *Am. Jour. Physiol.*, 69, 371, 1924.
[2] Morgulis: *Jour. Biol. Chem.*, 66, 353, 1926.



The biuret may be dissolved in water and a reddish-violet color obtained by treating the aqueous solution with copper sulphate and potassium hydroxide (see Biuret Test, page 164). Certain hypochlorites or hypobromites in alkaline solution have the power of decomposing urea into nitrogen, carbon dioxide, and water. Sodium hypobromite brings about this decomposition, as follows:

$$CO(NH_2)_2+3NaOBr\rightarrow 3NaBr+N_2+CO_2+2H_2O.$$

According to the Werner hypothesis[1] the urea is momentarily brominated and the resultant compound hydrolyzed by the alkali and oxidized by hypobromite. Sodium cyanate, sodium nitrate, hydrazine and CO are formed as by-products thus accounting for the low nitrogen values obtained in the clinical application of this method.

The soy bean and jack bean have been shown to contain an enzyme called *urease* which has the power to decompose urea with the liberation of ammonia.[2] This fact is made use of in the quantitative determination of urea (see Chapter XXXI).

[1] Werner: *The Chemistry of Urea*, Longmans, Green & Co., London, 1923.
[2] Takeuchi: *Jour. College of Agr.*, Tokyo, 1909, Part I.

Urea has the power of forming crystalline compounds with certain acids; urea nitrate and urea oxalate are the most important of these compounds. *Urea nitrate*, $CO(NH_2)_2.HNO_3$, crystallizes in colorless,

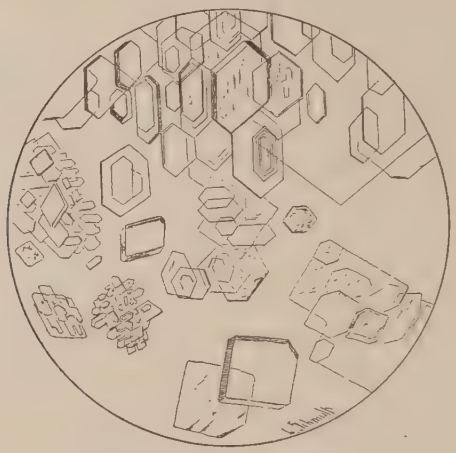


FIG. 185.—UREA NITRATE.

rhombic or six-sided tiles (Fig. 185, above), which are easily soluble in water. *Urea oxalate*, $(CO(NH_2)_2)_2.H_2C_2O_4$, crystallizes in the form of rhombic or six-sided prisms or plates (Fig. 187, page 600): the oxalate differs from the nitrate in being somewhat less soluble in water. The formation of the nitrate and oxalate and the decomposition of urea by the enzyme urease are the most satisfactory methods for the detection of urea.

A decrease in the excretion of urea is observed in many diseases in which the diet is much reduced and in some disorders as a result of alterations in metabolism, *e.g.*, myxedema, and in others as a result of changes in excretion, as in severe and advanced kidney disease. A pathological increase is found in a large proportion of diseases which are associated with a toxic state. In marked acidosis it may be considerably decreased relative to the total nitrogen (see Ammonia).

EXPERIMENTS ON UREA

1. Isolation from the Urine.[1]**—Place 800 c.c. of urine in a precipitating jar add 250 c.c. of baryta mixture,**[2] **and stir thoroughly. Filter off the precipitate**

[1] The method based upon the precipitation by nitric acid is also satisfactory (see Hoppe-Seyler's *Handbuch der Physiol. und Pathol. Chem. Anal.*, Eighth edition, 1909, p. 145).

[2] Baryta mixture consists of a mixture of 1 volume of a saturated solution of $Ba[NO_3]_2$ and 2 volumes of a saturated solution of $Ba[OH]_2$.

of phosphates, sulphates, urates, and hippurates and evaporate the filtrate on a water-bath to a thick syrup. This syrup contains chlorides, creatinine, organic salts, pigments, and urea. Extract the syrup with warm 95 per cent alcohol and filter again. The filtrate contains the urea contaminated with pigment. Decolorize the filtrate by boiling with animal charcoal, filter again, and stand the filtrate away in a cold place for crystallization. Examine the crystals under the microscope and compare them with those shown in Fig. 184, page 595.

2. Solubility.—Test the solubility of urea, prepared by yourself or furnished by the instructor, in water and in alcohol and ether.

3. Melting-point.—Determine the melting-point of some pure urea furnished by the instructor. Proceed as follows: Into an ordinary melting-point tube, sealed at one end, introduce powdered urea. Fasten the tube to the bulb of a thermometer as shown in Fig. 126, and suspend the bulb and its attached tube in a small beaker containing sulphuric acid. Gently raise the temperature of the acid by means of a low flame, stirring the fluid continually, and note the temperature at which the urea begins to melt.

4. Crystalline Form.—Dissolve a crystal of pure urea in a few drops of 95 per cent alcohol and place 1–2 drops of the alcoholic solution on a microscopic slide. Allow the alcohol to evaporate spontaneously, examine the crystals under the microscope, and compare them with those reproduced in Fig. 184, page 595. Recrystallize a little urea from water in the same way and compare the crystals with those obtained from the alcoholic solution.

Fig. 186.—Melting-point Tubes Fastened to Bulb of Thermometer.

5. Formation of Biuret.—Place a small amount of urea in a dry test-tube and heat carefully in a low flame. The urea melts at 132°C. and liberates ammonia. Continue heating until the fused mass begins to solidify. Cool the tube, dissolve the residue in dilute potassium hydroxide solution, and add very dilute copper sulphate solution (see page 164). The purplish-violet color is due to the presence of biuret which has been formed from the urea through the application of heat as indicated. The chemistry of this reaction is shown on p. 164.

6. Urea Nitrate.—Prepare a concentrated solution of urea by dissolving a little of the substance in a few drops of water. Place a drop of this solution on a microscopic slide, add a drop of concentrated nitric acid, and examine under the microscope. Compare the crystals with those reproduced in Fig. 185, page 598.

7. Urea Oxalate.—To a drop of a concentrated solution of urea, prepared as described in the last experiment (6), add a drop of a saturated solution of oxalic acid. Examine under the microscope and compare the crystals with those shown in Fig. 187, page 600.

8. Decomposition by Urease.—To 5 c.c. of urea solution in a test tube add 1 c.c. of urease solution or a little soy bean or jack bean powder. Allow the tube to stand for ten minutes, heat the contents to boiling, holding moist red

and blue litmus papers at the mouth of the tube. What do you observe? Note the odor. Explain.

9. Decomposition by Sodium Hypobromite.—Into a mixture of 3 c.c. of concentrated sodium hydroxide solution and 2 c.c. of bromine water in a test-tube introduce a crystal of urea or a small amount of concentrated solution of urea. Through the influence of the sodium hypobromite, NaOBr, the urea is decomposed and carbon dioxide and nitrogen are liberated. The carbon dioxide is absorbed by the excess of sodium hydroxide, while the nitrogen is evolved and causes the marked effervescence observed. This property forms the basis for one of the methods in common use for the quantitative determination of urea. Write the equation showing the decomposition of urea by sodium hypobromite.

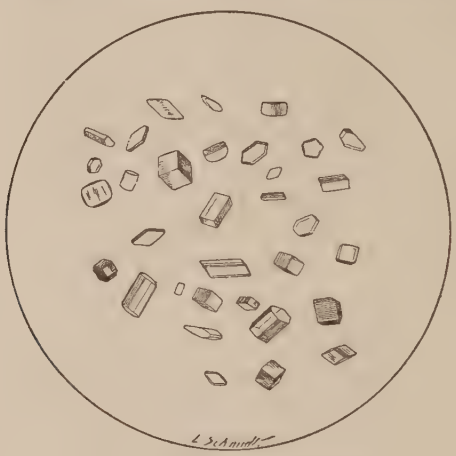
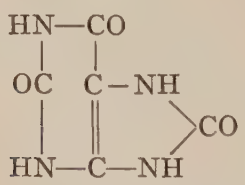


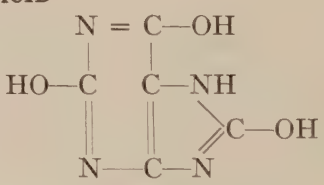
FIG. 187.—UREA OXALATE.

It is claimed that all ammonium compounds and all compounds containing the amino [$-NH_2$] group yield nitrogen when treated with hypobromite as in this test.

URIC ACID[1]



Keto (lactam) form



Enol (lactim) form

Uric acid is one of the most important of the constituents of the urine. It is generally stated that normally about 0.7 gram is excreted in 24 hours, but that this amount is subject to wide variations, particularly under certain dietary and pathological conditions. It has been shown, however, that the average daily excretion of uric acid for ten men ranging in age from 19 to 29 years and fed a normal mixed diet

[1] For review on purine metabolism see Rose: *Physiol. Reviews:* 3, 544, 1923.

was 0.597 gram, a value somewhat lower than the generally accepted average of 0.7 gram for such a period.[1] On a *purine-free* diet the uric acid output may be 0.1–0.5 gram per day, whereas a *high purine* diet may yield a daily output of 2 grams. Uric acid is a diureide and consequently upon oxidation may yield two molecules of urea. It acts as a weak dibasic acid and forms two classes of salts, neutral and acid. The neutral potassium and lithium urates are the most easily soluble of the alkali salts; the ammonium urate is difficultly soluble. The acid-alkali urates are more insoluble and form the major portion of the sediment which separates upon cooling the concentrated urine; the alkaline earth urates are very insoluble. Ordinarily uric acid occurs in the urine in the form of urates and upon acidifying the liquid the uric acid is liberated and deposits in crystalline form. This property forms the basis of one of the older methods for the quantitative determination of uric acid (Heintz Method.)

Uric acid is very closely related to the purine bases as may be seen from a comparison of its structural formula with those of the purine bases given on page 192. According to the purine nomenclature it is designated 2–6–8-trioxypurine. Uric acid forms the principal end-product of the nitrogenous metabolism of birds and scaly reptiles; in the human organism it occupies the fourth position inasmuch as here urea, ammonia, and creatinine are the chief end-products of nitrogenous metabolism. It is generally said that the relation existing between uric acid and urea in human urine under normal conditions varies on the average from 1:40 to 1:100 and is subject to wider variations under pathological conditions; and further that because of the high content of uric acid in the urine of newborn infants the ratio may be increased to 1:10 or even higher. We now know that this ratio of uric acid to urea is of little significance under any conditions.

In man, uric acid probably results principally from the destruction of nuclein material. It may arise from nuclein or other purine material ingested as food or from the disintegrating cellular matter of the organism. The uric acid resulting from the first process is said to be of *exogenous* origin, whereas the product of the second form of activity is said to be of *endogenous* origin. As a result of experimentation, Sivén, and Burian and Schur, and Rockwood claim that the amount of endogenous uric acid formed in any given period is fairly constant for each individual under normal conditions, and that it is entirely independent of the total amount of nitrogen eliminated. Folin has taken exception to the statements of these investigators and claims that, following a pronounced decrease in the amount of protein metabolized, the absolute

[1] Hanzlik and Hawk: *Jour. Biol. Chem.*, 5, 355, 1908.

quantity of uric acid is decreased but that this decrease is relatively smaller than the decrease in the total nitrogen excretion and that the per cent of the uric acid nitrogen, in terms of the total nitrogen, is therefore decidedly increased. According to Mareš,[1] food-stuffs act to increase the endogenous uric acid output by stimulating the digestive glands to activity. That a portion of the endogenous uric acid may arise in this way has recently been shown by Mendel and Stehle.[2] It is further possible that some of it arises from the amino-acids arginine and histidine.[3]

From experiments on dogs, Mann and associates[4] draw the conclusion that the destruction of uric acid depends on the presence of the liver. These experiments also indicate that the liver is of considerable importance in the metabolism of the purins.

In birds the formation of uric acid is analogous to the formation of urea in man. In these organisms it is derived principally from the protein material of the tissues and the food and is formed through a process of synthesis which occurs for the most part in the liver; a comparatively small fraction of the total uric acid excretion of birds may result from nuclein material.

When pure, uric acid may be obtained as a white, odorless, and tasteless powder, which is composed principally of small, transparent, crystalline, rhombic plates. Uric acid as it separates from the urine is invariably pigmented, and crystallizes in a large variety of characteristic forms, *e.g.*, dumb-bells, wedges, rhombic prisms, irregular rectangular or hexagonal plates, whetstones, prismatic rosettes, etc. Uric acid is insoluble in alcohol and ether, soluble with difficulty in boiling water (1 : 1800) and practically insoluble in cold water (1 : 39,480, at 18°C.). It is soluble in alkalis, alkali carbonates, boiling glycerol, concentrated sulphuric acid, and in certain organic bases such as ethylamine and piperidine. It is claimed that the uric acid is held in solution in the urine by the urea and disodium hydrogen phosphate present. Uric acid possesses the power of reducing cupric hydroxide in alkaline solution and may thus lead to an erroneous conclusion in testing for sugar in the urine by means of Fehling's or Trommer's test. A white precipitate of cuprous urate is formed if only a small amount of cupric hydroxide is present, but if enough of the copper salt is present the characteristic red or brownish-red precipitate of cuprous oxide is obtained. Uric acid does not possess the power of reducing bismuth in

[1] Mareš: *Arch. f. d. ges. Physiol.*, 134, 59, 1910.
[2] Mendel and Stehle: *Jour. Biol. Chem.*, 22, 215, 1915.
[3] Rose: Jour. Biol. Chem., 48, 563, 1921.
[4] Mann: *Jour. Am. Med. Assn.*, 85, 1472, 1925.

PLATE VI.

Uric Acid Crystals. Normal Colors. (From *Purdy*, after *Peyer*.)

alkaline solution and therefore does not interfere in testing for sugar in the urine by means of Boettger's or Nylander's tests.

In addition to being an important urinary constituent uric acid is present in small amounts in normal human blood as well as in the blood of birds. It is also normally present in the brain, heart, liver, lungs, pancreas, and spleen.

Pathologically, the excretion of uric acid is subject to wide variations, but the experimental findings are rather contradictory. It may be stated with certainty, however, that in leukemia, because of the destruction of nuclein material, the uric acid output is increased absolutely as well as relatively to the urea output; under these conditions the ratio between the uric acid and urea may be as low as 1:9, whereas the normal ratio, as we have seen, is 1:50 or higher. An actual output of 12 grams of uric acid per day has been reported in leukemia. In the study of the influence of X-ray on metabolism Edsall and others have reached some interesting conclusions. Edsall found that the excretion of uric acid is usually increased and that in some conditions, particularly in leukemia, it may be *greatly* increased. The excretion of total nitrogen, phosphates, and other sustances may also be considerably increased.

In gout the kidney is said to lose the power of properly eliminating uric acid and it collects in the blood in abnormally high concentration.

Normal = 2–3.5 mg. uric acid per 100 grams of blood.
Gout = 4–10 mg. uric acid per 100 grams of blood.

In gout the uric acid content of the urine is generally low preceding an attack and increases during the attack. Atophan and salicylates have been found to increase the uric acid output in gout, apparently due to increased kidney activity.

The extirpation of the liver causes an accumulation of uric acid in the blood and tissues or an increased elimination in the urine, if renal activity is maintained.[1]

The uric acid content of the urine is of importance in relation to the formation of uric acid calculi. The administration of alkali carbonates and citrates, or the feeding of base-forming foods, by decreasing the acidity of the urine increases its solvent power for uric acid and decreases the liability of formation of this type of calculus.[2]

Experiments on Uric Acid

1. Isolation from the Urine.—Place about 200 c.c. of filtered urine in a beaker, render it acid with 2–10 c.c. of concentrated hydrochloric acid, stir

[1] Mann: *Jour. Am. Med. Assn.*, 85, 1472, 1925.
[2] Blatherwick: *Arch. Int. Med.*, 14, 409, 1914.

thoroughly, and stand the vessel in a cold place for 24 hours. Examine the pigmented crystals of uric acid under the microscope and compare them with those shown in Fig. 205, page 684, and Pl. VI, opposite page 602.

2. Solubility.—Try the solubility of pure uric acid, furnished by the instructor, in water, dilute acid and alkali and in alcohol, ether and concentrated sulphuric acid.

3. Crystalline Form of Pure Uric Acid.—Place about 100 c.c. of water in a small beaker, render it distinctly alkaline with potassium hydroxide solution and

FIG. 188.—PURE URIC ACID.

add a small amount of pure uric acid, stirring continuously. Cool the solution, render it distinctly acid with hydrochloric acid and allow it to stand in a cool place for crystallization. Examine the crystals under the microscope and compare them with those reproduced in Fig. 188.

4. Murexide Test.—To a small amount of pure uric acid in a small evaporating dish add 2–3 drops of concentrated nitric acid. Evaporate to dryness carefully on a water-bath or over a very low flame. A red or yellow residue remains which turns purplish red after cooling the dish and adding a drop of very dilute ammonium hydroxide. The color is due to the formation of murexide. If potassium hydroxide is used instead of ammonium hydroxide a purplish violet color due to the production of the potassium salt is obtained. The color disappears upon warming; with certain related bodies (purine bases) the color persists under these conditions.

In this reaction the uric acid is oxidized to dialuric acid and alloxan. These two substances condense to form alloxantin. This alloxantin reacts with ammonium hydroxide to form purpuric acid. The purple color is due to the formation of *ammonium purpurate* or *murexide*.

5. Phosphotungstic Acid Reaction (Folin).—To 20 c.c. of saturated sodium carbonate solution in a small beaker add a small amount of uric acid. Stir the solution until the uric acid has dissolved, then add 1 c.c. of Folin's uric acid reagent (see Chapter XXXI). A blue color results.

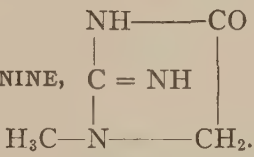
6. **Silver Reduction Test (Schiff).**—Dissolve a small amount of pure uric acid in sodium carbonate solution and transfer a drop of the resulting mixture to a strip of filter paper saturated with silver nitrate solution. A yellowish-brown or black coloration due to the formation of reduced silver is produced.

It is claimed that chlorides interfere with this test.

7. Influence upon Fehling's and Benedict's Solutions.—Dilute 1 c.c. of Fehling's solution with 4 c.c. of water and heat to boiling. Now add slowly, a few drops at a time, 1–2 c.c. of a concentration solution of uric acid in potassium hydroxide, heating after each addition. From this experiment what do you conclude regarding the possibility of arriving at an erroneous decision when testing for sugar in the urine by means of Fehling's test? Repeat using Benedict's solution.

8. **Reduction of Nylander's Reagent.**—To 5 c.c. of a solution of uric acid in potassium hydroxide add about one-half a cubic centimeter of Nylander's reagent and heat to boiling for a few moments. Do you obtain the typical black end-reaction signifying the reduction of the bismuth?

CREATININE,



Creatinine is the anhydride of creatine and is a constituent of normal human urine. The theory that creatinine is derived from the creatine of ingested muscular tissue as well as from the creatine of the muscular tissue of the organism has been proven to be incorrect by Folin, Klercker, and Wolf and Shaffer. Shaffer believes that creatinine is the result of some special process of normal metabolism which takes place to a large extent, if not entirely, in the muscles, and further that the amount of such creatinine elimination, expressed *in milligrams per kilogram body weight*, is an index of this special process.[1] He further states that the muscular efficiency of the individual depends upon the intensity of this process. Under normal conditions about 1–1.25 grams of creatinine is excreted by an adult man in 24 hours,[2] the exact amount depending in great part upon the nature of the food and decreasing markedly in starvation. Very little that is important is known regarding the excretion of creatinine under pathological conditions. The creatinine content of the urine is said to be increased in typhoid fever, typhus, tetanus, and pneumonia, and to be decreased in anæmia, chlorosis, paralysis, muscular atrophy, advanced degeneration of the kidneys,

[1] He proposes to designate as the "creatinine coefficient" the excretion of *creatinine-nitrogen (mg.) per kilogram of body weight.*

[2] According to Shaffer the amount excreted by strictly normal individuals is between 7 and 11 mg. of creatinine-nitrogen per kilogram of body weight.

and in leukemia (myelogeneous, lymphatic and pseudo). An increase of creatinine was also noted in diabetes, an increase probably due to the creatinine content of the meat eaten. The greater part of the data, however, relating to the variation of the creatinine excretion under pathological conditions are not of much value since in nearly every instance the diet was not sufficiently controlled to permit the collection of reliable data. And further, until the advent of the Folin method (see page 734) there was no accurate method for the quantitative determination of creatinine. Shaffer has called attention to the fact that a low excretion of creatinine is found in the urine of a remarkably large number of pathological subjects, representing a variety of con-

FIG. 189.—CREATININE.

ditions, and that it is therefore evident that the excretion of an abnormally small amount of this substance is by no means peculiar to any one disease. A considerable increase in the creatinine content of the blood is observed in uremia.[1]

According to Mann and associates[2] the changes in the blood creatinine after removal of the liver depend entirely on the kidneys. If the kidneys are active the blood creatinine does not change. If the kidneys are removed as well, or the animal becomes anuric, the creatinine increases in the blood at the same rate as when only the kidneys are removed. The creatine behaves in a similar manner. The liver would therefore appear to take no specific part in the creatinine-creatine metabolism.

[1] **Folin and Denis:** *Jour. Biol. Chem.*, 17, 487, 1914.
Myers: *Practical Chemical Analysis of Blood*, Second Edition, 1925.
[2] Mann: *Jour. Am. Med. Assn.*, 85, 1472, 1925.

Creatinine crystallizes in colorless, glistening monoclinic prisms (Fig. 189, page 606) which are soluble in about 12 parts of cold water; they are more soluble in warm water and in warm alcohol. It forms salts only with strong mineral acids. One of the most important and interesting of the compounds of creatinine is *creatinine-zinc chloride*, $(C_4H_7N_3O)_2$-$ZnCl_2$, which is formed from an alcoholic solution of creatinine upon treatment with zinc chloride in acid solution. Creatinine has the power of reducing cupric hydroxide in alkaline solution and in this way may interfere with the determination of sugar in the urine. In the reduction by creatinine the blue liquid is first changed to a yellow, and the formation of a brownish-red precipitate of cuprous oxide is brought about only after continuous boiling with an excess of the copper salt. Creatinine does not reduce alkaline bismuth solutions and therefore does not interfere with Nylander's and Boettger's tests.

It has been shown by Folin that the absolute quantity of creatinine eliminated in the urine on a meat-free diet is a constant quantity different for different individuals, but wholly independent of quantitative changes in the total amount of nitrogen eliminated. Shaffer has confirmed these findings and has shown that the output of creatinine under these conditions is constant from hour to hour as well as from day to day.

Experiments on Creatinine and Creatine

1. Preparation of Pure Creatinine from Urine (Folin-Benedict[1]).—To 10 liters[2] of undecomposed urine in a large precipitating jar add with stirring a hot solution of 180 grams of picric acid in 450 c.c. of boiling alcohol. Allow to stand over night and syphon off the supernatant fluid. Pour the residue upon a large Buchner funnel, drain with suction, wash once or twice with cold saturated picric acid and suck dry. Treat the dry or nearly dry picrate in a large mortar or evaporating dish with enough concentrated HCl to form a moderately thin paste (about 60 c.c. of acid for each 100 grams of picrate) and stir the mixture thoroughly with the pestle for 3–5 minutes. Filter with suction on a hardened paper, and wash the residue twice with enough water to cover it, sucking as nearly dry as possible each time. Transfer the filtrate to a large flask and neutralize with an excess of solid magnesium oxide (the "heavy" variety is best). Add this oxide in small portions with cooling of the flask under running water between the additions. Neutralization of the acid will be indicated by a bright yellow color of the mixture, or litmus paper may be used to test it. Filter with suction. Wash the residue twice with water. Immediately add a few cubic centimeters of glacial acetic acid to the filtrate to make it strongly acid. Pay no attention to any precipitate that may form, but dilute the solution with about 4 volumes of 95 per cent alcohol. After 15 minutes filter off the slight precipitate which forms. Treat the final filtrate with 30–40 c.c. of 30 per cent zinc chloride. Stir and let

[1] Benedict: *Jour. Biol. Chem.*, 18, 182, 1914.
Folin: *Ibid.*, 17, 463, 1914.

[2] If it is simply desired to demonstrate the presence of creatinine, 1 liter may be employed and the various reagents reduced accordingly.

stand over night in a cool place. Pour off the supernatant liquid and collect the creatinine zinc chloride on a Buchner funnel, wash once with water, then thoroughly with 50 per cent alcohol, finally with 95 per cent alcohol and dry. A nearly white, light crystalline powder should be obtained. The yield should be 90–95 per cent of the original creatinine (usually about 1.5–1.8 grams of creatinine zinc chloride per liter of urine).

Recrystallize the creatinine-zinc chloride by treating 10 grams with 100 c.c. of water and about 60 c.c. of normal sulphuric acid, heating the mixture until a clear solution is obtained. Add about 4 grams of purified animal charcoal, continue boiling for about a minute, filter with suction through a small Buchner funnel, pouring the filtrate back on the filter three or four times until it runs through perfectly colorless. Wash residue with hot water and transfer the total filtrate to a beaker and while hot treat with a little strong zinc chloride solution

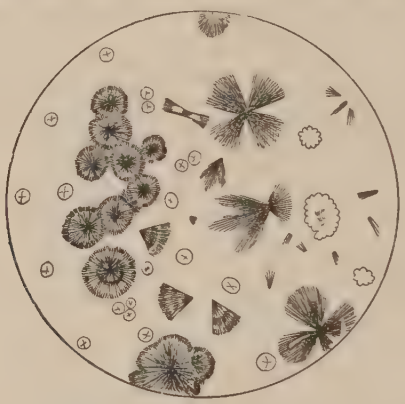


FIG. 190.—CREATININE-ZINC CHLORIDE. (*Salkowski.*)

(3 c.c.) and with about 7 grams of potassium acetate dissolved in a little water. After ten minutes dilute with an equal volume of alcohol, and allow to stand in a cold place for some hours. Filter off the crystalline product and examine under microscope (see Fig. 190). To remove the small amount of potassium sulphate which it contains stir up with twice its weight of water, filter, wash with a little water and then with alcohol. The preparation should be snow white. Yield, 85–90 per cent.

For the decomposition of the creatinine zinc chloride Gaebler[1] suggests the following modification of the original procedure. Place 32 gm. creatinine zinc chloride in a pressure bottle (a citrate of magnesia bottle will serve) and add 225–250 c.c. conc. ammonia. Close the bottle and heat in a water bath at 70–80° shaking to effect solution. Cool quite rapidly to room temperature and then in a salt-ice bath. Pure creatinine crystallizes out. Filter. Wash with ice cold ammonia, then with acetone and dry. The yield should be 75 per cent (15 gm. creatinine). The product is perfectly pure and can be used as a standard in the quantitative determination of creatine and creatinine. See chapters on Quantitative Analysis of Urine and Blood.

The yield in this process is about 18 grams of recrystallized creatine, and about 55 grams of creatinine zinc chloride recovered. Longer boiling with lime does not bring about a greater yield, as after the 20-minute point creatine is decomposed almost exactly as fast as it is formed.

[1] Gaebler: Personal communication, 1926.

Examine the crystals of creatine under the microscope and compare with illustration in Chapter XXIV on Muscular Tissue. For other creatine tests see Chapter XXIV.

2. **Nitro-prusside Test (Weyl).—Take 5 c.c. of urine in a test-tube, add a few drops of sodium nitro-prusside and render the solution alkaline with potassium hydroxide solution. A ruby-red color results which soon turns yellow. See Legal's test for acetone, page 658.**

3. **Nitro-prusside-acetic Acid Test (Salkowski).**—To the yellow solution obtained in Weyl's test above add an excess of acetic acid and apply heat. A green color results and is in turn displaced by a blue color. A precipitate of Prussian blue may form.

4. **Picric Acid Reaction (Jaffe).—Place 5 c.c. of urine in a test-tube, add an aqueous solution of picric acid and render the mixture alkaline with potassium hydroxide solution. A red color is produced which turns yellow if the solution be acidified. Glucose gives a similar red color but only upon the application of heat. This color reaction observed when creatinine in alkaline solution is treated with picric acid is the basic principle of Folin's colorimetric method for the quantitative** determination of creatinine (see page 734) and is due to the formation of a red tautomer of creatinine picrate.[1] The production of this tautomer is "dependent upon the formation of a salt, a keto-enol change within the creatinine molecule, and a change in the picric acid molecule involving the hydrogens in the meta positions and, probably, all three nitro groups.[2]

5. **Preparation of Creatine.**—Creatine may be prepared from creatinine zinc chloride by decomposition with calcium hydrate, the process being one of hydrolysis (Benedict).

One hundred grams of creatinine zinc chloride are treated with about 700 c.c. of water in a large casserole and the mixture heated to boiling; 150 grams of pure powdered calcium hydrate are then added, with stirring, and the mixture boiled gently for 20 minutes (with occasional stirring). The hot mixture is then filtered with suction, the residue being washed with hot water. The filtrate is then treated with hydrogen sulphide gas for a few minutes and poured through a folded filter to remove the zinc. The filtrate is acidified by the addition of about 5 c.c. of glacial acetic acid and boiled down rapidly to a volume of about 200 c.c. This solution is allowed to stand over night, preferably in a cool place. The next day the crystallized creatine is filtered off with suction, washed with a very little cold water, and then thoroughly washed with alcohol and dried.[3] This product is then recrystallized by dissolving in about seven times its weight of boiling water and allowing the solution to cool slowly and stand for some hours. This product should be perfectly pure creatine. If necessary it can be recrystallized with very little loss. The crystallized product should be filtered off, washed with alcohol and ether and dried in air for about half an hour. Thus obtained the creatine contains water of crystallization which it loses very readily upon exposure to air. To prepare creatine which can be weighed with absolute exactness it is necessary to dehydrate this product by heating for some hours at about 95°.

ETHEREAL SULPHATES

The most important of the ethereal sulphates found in the urine are *phenol-sulphuric acid*, *p-cresol-sulphuric acid*, *indoxyl-sulphuric*

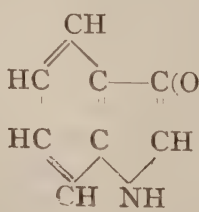
[1] Greenwald and Gross: *Jour. Biol. Chem.*, 59, 601, 1924.

[2] Greenwald: *Jour. Am. Chem. Soc.*, 47, 1443, 1925.

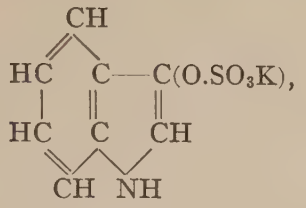
[3] The filtrate obtained at this point should be diluted with alcohol and treated with zinc chloride (50 c.c. of a 30 per cent solution) for recovery of the unconverted creatinine.

acid, and *skatoxyl-sulphuric acid*. Pyrocatechol-sulphuric acid also occurs in traces in human urine. The total output of ethereal sulphuric acid (as SO_3) varies ordinarily from 0.1 gram to 0.25 gram for 24 hours and comprises 5–15 per cent of the total sulphur. In health the ratio of ethereal sulphuric acid to inorganic sulphuric acid is about 1:10. These ethereal sulphuric acids originate in part from the phenol, cresol, indole and skatole formed in the putrefaction of protein material in the intestine. The phenol passes to the liver where part of it is conjugated to form phenol potassium sulphate and appears in this form in the urine whereas the indole and skatole undergo a preliminary oxidation to form *indoxyl* and *skatoxyl* respectively before their conjugation and elimination.

It was formerly generally considered that each of the ethereal sulphuric acids was formed principally in the putrefaction of protein material in the intestine and that therefore a determination of the total ethereal sulphuric acid content of the urine was an index of the extent to which these putrefactive processes were proceeding within the organism. Folin, however, conducted a series of experiments which seemed to show that the ethereal sulphuric acid content of the urine did *not* afford an index of the extent of intestinal putrefaction, since these bodies arise only in part from putrefactive processes. He claims that the ethereal sulphuric acid excretion represents a form of sulphur metabolism which is more in evidence upon a diet containing a very small amount of protein or upon a diet containing absolutely no protein. The ethereal sulphuric acid content of the urine diminishes as the total sulphur content diminishes but the *percentage decrease* is much less. Therefore when considered from the standpoint of the total sulphuric acid content the ethereal sulphuric acid content is not diminished but is *increased*, although the total sulphuric acid content *is diminished*. Folin's experiments also seem to show that the indoxyl sulphuric acid (indoxyl potassium sulphate or indican) content of the urine does not originate to any degree from the metabolism of protein material but that it arises in great part from intestinal putrefaction and that the excretion of indoxyl sulphuric acid may *alone* be taken as a rough index of the extent of putrefactive processes within the intestine. Indoxyl sulphuric acid,



therefore, which occurs in the urine as indoxyl potassium sulphate or indican,



is clinically the most important of the ethereal sulphuric acids. Under normal conditions from 4 to 20 mg. of indican are excreted per day. The variations are due mainly to diet, a high meat diet causing an increase and a carbohydrate diet a decrease. Pathologically the greatest increases are found in disorders involving increased putrefaction and stagnation of intestinal contents. Bacterial decomposition of body protein as in gangrene, putrid pus formation, etc., gives rise to an increased indican excretion.

It was formerly believed that the *phenol* was excreted practically quantitatively in the conjugated form. Researches of Folin and Denis[1] seem to indicate that this is not true. Only *part* of the phenols formed in intestinal putrefaction are excreted in the conjugated form, the remainder being excreted as free phenol. The phenol output tends to vary directly but not proportionally with the protein ingestion. The total phenol excretion of normal men on an ordinary mixed diet averages around 0.4 gram per day.

Tests for Indican[2]

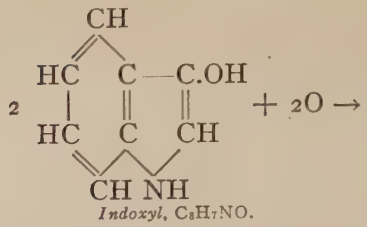
1. Jaffe's Test.—Nearly fill a test-tube with a mixture composed of equal volumes of concentrated HCl and the urine under examination. Add 2–3 c.c. of chloroform and a few drops of a calcium hypochlorite solution, place the thumb over the end of the test-tube and shake the tube and contents thoroughly. The chloroform is colored more or less, according to the amount of indican present. Ordinarily a blue color due to the formation of indigo-blue is produced; less frequently a red color due to indigo-red may be noted.

Repeat this test on some of this same urine to which formaldehyde has been added. Is there any variation in the reaction from what you previously obtained?

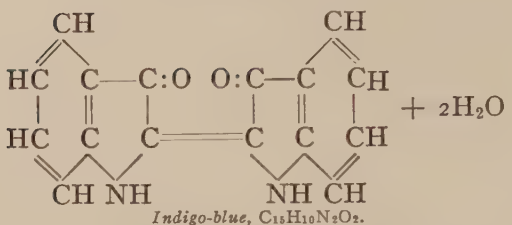
The following represents the reaction (see also page 301):

[1] Folin and Denis: *Jour. Biol. Chem.*, 22, 309, 1915.

[2] The urine should always be examined *fresh* if this is possible. In any event formaldehyde should never be used as a preservative for such urines as are to be examined for indican by means of any test involving hypochlorite or potassium permanganate. The formaldehyde through its reducing power lowers the oxidizing efficiency of the mixture. The formation of formic acid from the aldehyde may also interfere.



Indoxyl, C_8H_7NO.



Indigo-blue, $C_{16}H_{10}N_2O_2$.

2. Obermayer's Test.—Nearly fill a test-tube with a mixture composed of equal volumes of Obermayer's reagent[1] and the urine under examination. Add 2–3 c.c. of chloroform,[2] place the thumb over the end of the test-tube and shake thoroughly. How does this compare with Jaffe's test?

3. Jolles' Reaction.[3]—To 10 c.c. of urine add 1 c.c. of a 5 per cent alcoholic thymol solution and shake. Add about 10 c.c. of fuming HCl containing 5 grams of ferric chloride per liter. Shake again carefully and let stand for 15 minutes. Add about 4 c.c. of chloroform and extract the pigment by repeated gentle shaking. The chloroform becomes intensely violet. 0.0032 mg. of indican can be detected in 10 c.c. of urine. It is much the most delicate test for indican.

HIPPURIC ACID, $C_6H_5.CO.NH.CH_2.COOH$.

This acid occurs normally in the urine of both the carnivora and herbivora but is much more abundant in the urine of the latter. It is formed by a synthesis of benzoic acid and glycocoll which takes place in the kidneys and elsewhere.[4] The glycocoll comes from decomposition of protein. The benzoic acid thus utilized may come from (1) preformed benzoic acid of fruits and vegetables; (2) other aromatic compounds of fruits and vegetables; (3) aromatic amino-acids (tyrosine and phenylalanine) from the alimentary tract. The average excretion of hippuric acid by an adult man for 24 hours under normal conditions is about 0.7 gram. Hippuric acid crystallizes in needles or rhombic prisms (see Fig. 191, p. 613) the particular form depending upon the

[1] Obermayer's reagent is prepared by adding 2–4 grams of ferric chloride to a liter of concentrated HCl (sp. gr. 1.19).

[2] Carbon disulphide may be substituted for chloroform if desired.

[3] *Zeit. physiol. Chem.*, 94, 79, 1915.

[4] Kingsbury and Bell: *Jour. Biol. Chem.*, 21, 297, 1915.
Lewis and Griffith: *Jour. Biol. Chem.*, 55, 22, 1923.

rapidity of crystallization. Pure hippuric acid melts at 187°C. The most satisfactory method for the isolation of hippuric acid from the urine in crystalline form is that proposed by Roaf (see below). It is easily soluble in alcohol or hot water, and only slightly soluble in ether. The output of hippuric acid is increased in diabetes owing probably to the ingestion of much protein and fruit. Plums, prunes and cranberries in particular increase the hippuric acid output considerably due to their relatively high content of benzoic acid. Hippuric acid is decreased in fevers and in certain kidney disorders where the synthetic

FIG. 191.—HIPPURIC ACID.

activity of the renal cells is diminished. It may be determined quantitatively by methods given in Chapter XXXI.

EXPERIMENTS ON HIPPURIC ACID

1. Separation from the Urine.—(a) First Method.—Render 500–1000 c.c of urine of the horse or cow[1] alkaline with milk of lime, boil for a few moments and filter while hot. Concentrate the filtrate, over a burner, to a small volume. Cool the solution, acidify it strongly with concentrated hydrochloric acid and stand it in a cool place for 24 hours. Filter off the crystals of hippuric acid which have formed and wash them with a little cold water. Remove the crystals from the paper, dissolve them in a very small amount of hot water and percolate the hot solution through thoroughly washed animal charcoal, being careful to wash out the last portion of the hippuric acid solution with hot water. Filter, concentrate

[1] If urine of the horse or cow is not available human urine may serve the purpose fully as well provided means are taken to increase its content of hippuric acid. This may be conveniently accomplished by ingesting 2 grams of ammonium benzoate at night. (See chapter on Metabolism.) The fraction of urine passed in the morning will be found to have a high content of hippuric acid. The ammonium benzoate is in no way harmful. In case ammonium benzoate is not available sodium benzoate may be substituted.

the filtrate to a small volume and stand it aside for crystallization. Examine the crystals under the microscope and compare them with those in Fig. 191, page 613. This method is not as satisfactory as Roaf's method (see below).

(b) Roaf's Method.—Place 500 c.c. of urine of the horse or cow in a casserole or precipitating jar and add an equal volume of a saturated solution of ammonium sulphate[1] and 7.5 c.c. of concentrated sulphuric acid. Permit the mixture to stand for 24 hours and remove the crystals of hippuric acid by filtration. Purify the crystals by recrystallization according to the directions given above under First Method. Examine the crystals under the microscope and compare them with those given in Fig. 191, page 613.

If sufficient urine is not available to permit the use of 500 c.c. a smaller volume may be used inasmuch as it is possible, by the above technic, to isolate hippuric acid in crystalline form from as small a volume as 25–50 c.c. of herbivorous urine. The greater the amount of ammonium sulphate added the more rapid the crystallization until at the saturation point the crystals of hippuric acid sometimes form in about ten minutes.

2. Melting-point.—Determine the melting-point of the hippuric acid prepared in the above experiment (see page 599).

3. Solubility.—Test the solubility of hippuric acid in hot and cold water and in alcohol, and ether.

4. Formation of Nitro-Benzene (Lücke's Reaction).—To a little hippuric acid in a small porcelain dish add 1–2 c.c. of concentrated HNO_3 and evaporate to dryness on a water-bath. Transfer the residue to a dry test-tube, apply heat, and note the odor of nitrobenzene (resembling that of oil of bitter almonds).

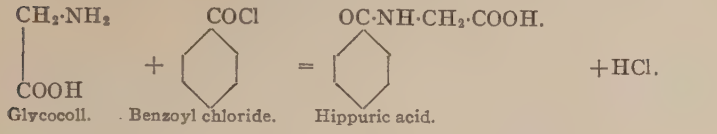
5. Sublimation.—Place a few crystals of hippuric acid in a dry test-tube and apply heat. The crystals are reduced to an oily fluid which solidifies in a crystalline mass upon cooling. When stronger heat is applied the liquid assumes a red color and finally yields a sublimate of benzoic acid and the odor of hydrocyanic acid.

6. Formation of Ferric Salt.—Render a small amount of a solution of hippuric acid neutral with dilute potassium hydroxide. Now add 1–3 drops of neutral ferric chloride solution and note the formation of the ferric salt of hippuric acid as a cream-colored precipitate.

7. Synthesis of Hippuric Acid.—To some of the glycocoll prepared in the last experiment or furnished by the instructor, add a little water, about 1 c.c. of benzoyl chloride and render alkaline with potassium hydroxide solution. Stopper the tube and shake it until no more heat is evolved. Now render strongly alkaline with potassium hydroxide and shake the mixture until no odor of benzoyl chloride can be detected. Cool, acidify with hydrochloric acid, add an equal volume of petroleum ether, and shake thoroughly to remove the benzoic acid. (Evaporate this solution and note the crystals of benzoic acid. Compare them with those shown in Fig. 193, page 620.) Decant the ethereal solution into a porcelain dish and extract again with ether. The hippuric acid remains in the aqueous solution. Filter it off and wash it with a small amount of cold water while still on the filter. Remove it to a small, shallow vessel, dissolve it in a small amount of hot water and set it aside for crystallization. Examine the crystals microscopically and compare them with those in Fig. 191, page 613.

[1] 125 grams of *solid* ammonium sulphate may be substituted.

The chemistry of the synthesis is represented thus:



OXALIC ACID, $\begin{matrix}\text{COOH}\\|\\\text{COOH}\end{matrix}$

Oxalic acid is a constituent of normal urine, about 15–20 mg. being eliminated in 24 hours. It is present in the urine as calcium oxalate which is kept in solution through the medium of the acid phosphates. The origin of the oxalic acid content of the urine is not well understood. When ingested it is eliminated, at least in part, unchanged, therefore since many of the common articles of diet, *e.g.*, asparagus, apples, cabbage, grapes, lettuce, rhubarb, spinach, tomatoes, etc., contain oxalic acid (oxalates) it seems probable that the ingested food supplies a portion of the oxalic acid found in the urine. There is also experimental evidence that part of the oxalic acid of the urine is formed within the organism in the course of protein and fat metabolism. It has also been suggested that oxalic acid may arise from an incomplete combustion of carbohydrates, especially under certain abnormal conditions. Pathologically, oxalic acid is found to be increased in amount in diabetes mellitus, in organic diseases of the liver, and in various other conditions which are accompanied by a derangement of the oxidation mechanism. An abnormal increase of oxalic acid is termed *oxaluria*. A considerable increase in the content of oxalic acid may be noted unaccompanied by any other apparent symptom. Calcium oxalate crystallizes in at least two distinct forms, *dumb-bells* and *octahedra* (Fig. 203, page 682).

Experiments

Preparation of Calcium Oxalate.—First Method.—Place 200–250 c.c. of urine in a beaker, add 5 c.c. of a saturated solution of calcium chloride, make the urine slightly acid with acetic acid, and stand the beaker aside in a cool place for 24 hours. Examine the sediment under the microscope and compare the crystalline forms with those shown in Fig. 203, page 682.

Second Method.—Proceed as above, replacing the acetic acid by an excess of ammonium hydroxide and filtering off the precipitate of phosphates.

NEUTRAL SULPHUR COMPOUNDS

Under this head may be classed such bodies as cystine (see page 142), oxyproteic acid, alloxyproteic acid, uroferric acid, methyl mercaptan, ethyl sulphide, thiocyanates and taurine derivatives. The sulphur

content of the bodies just enumerated is generally termed unoxidized or neutral sulphur in order that it may not be confused with the acid or oxidized sulphur which occurs in the inorganic sulphuric acid and ethereal sulphuric acid forms. Ordinarily the neutral sulphur content of normal human urine is 5–25 per cent of the total sulphur content (see "Sulphur Partition" in chapter on Metabolism). The actual amount excreted may be 0.2–0.4 grams per day, calculated as SO_3.

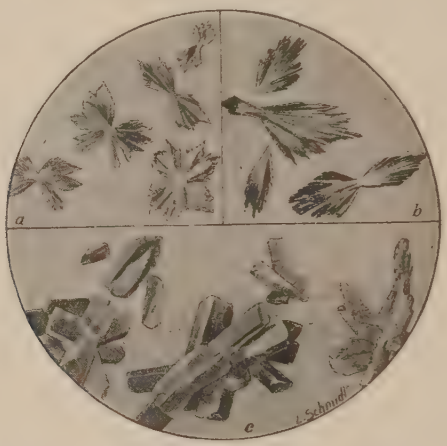
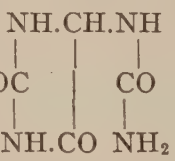


FIG. 192.—ALLANTOIN, FROM CAT'S URINE.
a and *b*, Forms in which it crystallized from the urine; *c*, recrystallized allantoin. (Drawn from micro-photographs furnished by Prof. Lafayette B. Mendel of Yale University.)

Its origin is mainly endogenous. The excretion is fairly constant for any given individual in spite of dietary changes. In tuberculosis, cancer, cystinuria, etc., the amount may be relatively or absolutely increased. (See page 173 for test for neutral sulphur.)

ALLANTOIN,



Allantoin is found in the urine of practically all mammals including man. In human urine it occurs in very small amount (5–15 mg. per day) whereas in the case of all other mammals investigated except anthropoid apes, it is the principal end-product of purine metabolism and may constitute 90 per cent or over of the total purine output.[1]

[1] Wiechowski: "Die Purinstoffe und das Allantoin" in Neubauer and Huppert's "*Analyse des Harns*," Wiesbaden, 1913.

Allantoin is formed by the oxidation of uric acid and the output is increased by the feeding of thymus or pancreas to lower animals. When pure it crystallizes in prisms (Fig. 192, page 616) and when impure in granules and knobs. Pathologically, it has been found increased in diabetes insipidus and in hysteria with convulsions (Pouchet). Mendel and Dakin[1] have shown that allantoin is optically inactive notwithstanding the fact that it contains an asymmetric carbon atom. This phenomenon they believe to be due to tautomeric change.

In the dog, according to Mann and associates,[2] the liver is the sole seat of uric acid destruction. After hepatectomy the uric acid excretion is equivalent to the allantoin output of the normal animal.

Experiments

1. Separation from the Urine.[3]—Meissner's Method.—Precipitate the urine with baryta water. Neutralize the filtrate carefully with dilute sulphuric acid, filter immediately, and evaporate the filtrate to incipient crystallization. Completely precipitate this warm fluid with 95 per cent alcohol (reserve the precipitate). Decant or filter and precipitate the solution by ether. Combine the ether and alcohol precipitates and extract with cold water or hot alcohol; allantoin remains undissolved. Bring the allantoin into solution in hot water and recrystallize.

2. Preparation from Uric Acid.—Dissolve 4 grams of uric acid in 100 c.c. of water rendered alkaline with potassium hydroxide. Cool and carefully add 3 grams of potassium permanganate. Filter, immediately acidulate the filtrate with acetic acid and allow it to stand in a cool place over night. Filter off the crystals and wash them with water. Save the wash water and filtrate, unite them and after concentrating to a small volume stand away for crystallization. Now combine all the crystals and recrystallize them from hot water. Use these crystals in the experiments which follow.

3. Microscopical Examination.—Examine the crystals made in the last experiment and compare them with those shown in Fig. 132.

4. Solubility.—Test the solubility of allantoin in cold and hot water, cold and hot alcohol and in ether.

5. Reaction.—Dissolve a crystal in water and test the reaction to litmus.

6. Furfural Test (Schiff).—Place a few crystals of allantoin on a test-tablet or in a porcelain dish and add 1–2 drops of a concentrated aqueous solution of furfural and 1–2 drops of concentrated hydrochloric acid. Observe the formation of a yellow color which turns to a light purple if allowed to stand. This test is given by urea but not by uric acid.

7. Murexide Test.—Try this test according to the directions given on page 604. Note that allantoin fails to respond.

8. Reduction of Benedict's Solution.—Make this test in the usual way (see page 76) except that the boiling must be prolonged and excessive. Ultimately

[1] Mendel and Dakin: *Jour. Biol. Chem.*, 7, 153, 1910.
[2] Mann: *Jour. Am. Med. Assn.*, 85, 1472, 1925.
[3] The urine of the dog after thymus, pancreas, or uric acid feeding may be employed,

the allantoin will reduce the solution. Compare with the result on uric acid, page 605.

AMINO-ACIDS

Certain of these acids are always present in normal urine. The excretion of total amino-acid nitrogen by a normal adult averages 0.4–1.0 gram per day or about 2–6 per cent of the total nitrogen. Free amino-acid nitrogen (for methods of estimation see page 728) is considerably less than this, and ordinarily constitutes 0.5–1 per cent of the total nitrogen. The amount may be largely increased in disorders associated with tissue waste, *e.g.*, typhoid, acidosis, pronounced atrophy of the liver, etc. After extirpation of the liver, urea formation ceases, and amino acids accumulate in the blood or are excreted in the urine if renal activity is maintained.[1] For tests on amino-acids see Chapter V.

AROMATIC OXYACIDS

Two of the most important of the oxyacids are *parahydroxy-phenyl-acetic acid*,

and *parahydroxy-phenyl-propionic acid*,

They are products of the putrefaction of protein material and tyrosine is an intermediate stage in their formation. Both these acids for the most part pass unchanged into the urine where they occur normally in very small amount. The content may be increased in the same manner as the phenol content, in particular by acute phosphorus poisoning. A fraction of the total aromatic oxyacid content of the urine is in combination with sulphuric acid, but the greater part is present in the form of salts of sodium and potassium.

Homogentisic Acid or di-hydroxyphenyl-acetic acid,

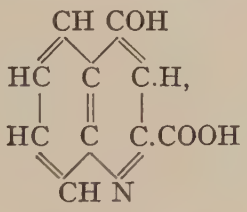
[1] Mann: *Jour. Am. Med. Assn.*, 85, 1472, 1925.

is another important oxyacid sometimes present in the urine. Under the name *glycosuric acid* it was first isolated from the urine by Prof. John Marshall of the University of Pennsylvania; subsequently Baumann isolated it and determined its chemical constitution. It occurs in cases of *alcaptonuria*. A urine containing this oxyacid turns greenish-brown from the surface downward when treated with a little sodium hydroxide or ammonia. If the solution be stirred the color very soon becomes dark brown or even black. Homogentisic acid reduces alkaline copper solutions but not alkaline bismuth solutions. Uroleucic acid is similar in its reaction to homogentisic acid.

Hydroxymandelic Acid or parahydroxyphenyl-glycolic acid,

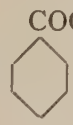
has been detected in the urine in cases of yellow atrophy of the liver.

Kynurenic Acid or γ-hydroxy-β-quinoline carboxylic acid,



is present in the urine of the dog and has recently been detected by Swain in the urine of the coyote. To isolate it from the urine proceed as follows: Acidify the urine with hydrochloric acid in the proportion 1:25. From this acid fluid both the uric acid and the kynurenic acid separate in the course of 24–48 hours. Filter off the combined crystalline deposit of the two acids, dissolve the kynurenic acid in dilute ammonia (uric acid is insoluble), and reprecipitate it with hydrochloric acid. If a solution containing kynurenic acid be evaporated to dryness with hydrochloric acid and potassium chlorate, a reddish residue is obtained which becomes first brownish green and then emerald green on adding ammonia (Jaffé).

Kynurenic acid may be quantitatively determined by Capaldi's method.[1]



BENZOIC ACID,

[1] *Zeitschrift für physiologische Chemie*, 23, 92, 1897.

Benzoic acid has been detected in the urine of the rabbit and dog. It is also said to occur in human urine accompanying renal disorders. The benzoic acid probably originates from a fermentative decomposition of the hippuric acid of the urine. Benzoic acid and glycocoll are synthesized in the kidney and elsewhere[1] to form hippuric acid (see page 614). Certain fruits and berries contain considerable benzoic acid; *e.g.*, *cranberries* have been shown to contain 0.06 per cent.[2]

Experiments

1. Solubility.—Test the solubility of benzoic acid in water, alcohol, and ether.

2. Crystalline Form.—Recrystallize some benzoic acid from hot water, examine the crystals under the microscope, and compare them with those reproduced in Fig. 193.

Fig. 193.—Benzoic Acid.

3. Sublimation.—Place a little benzoic acid in a test-tube and heat over a flame. Note the odor which is evolved and observe that the acid sublimes in the form of needles.

4. Dissolve a little sodium benzoate in water and add a solution of neutral ferric chloride. Note the production of a brownish-yellow precipitate (salicylic acid gives a reddish-violet color under the same conditions). Add ammonium hydroxide to some of the precipitate. It dissolves and ferric hydroxide is formed. Add a little hydrochloric acid to another portion of the original precipitate and stand the vessel away over night. What do you observe?

NUCLEOPROTEIN

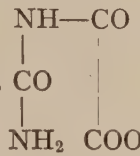
The nubecula of normal urine has been shown by one investigator to consist of a mucoid containing 12.7 per cent of nitrogen and 2.3 per cent of sulphur. This body evidently originates in the urinary passages. It is probably slightly soluble in the urine. Some investigators believe that the body forming the nubecula of normal urine is

[1] Kingsbury and Bell: *Jour. Biol. Chem.*, 21, 297, 1915.

[2] Radin [quoted by Blatherwick (*Arch. Int. Med.*, 14, 409, 1914) from unpublished data].

nucleoprotein and not a mucin or mucoid as stated above. A discussion of nucleoprotein and related bodies occurring in the urine under pathological conditions will be found on page 650.

OXALURIC ACID,



Oxaluric acid is not a constant constituent of normal human urine, and when found occurs only in traces as the ammonium salt. Upon boiling oxaluric acid it splits into oxalic acid and urea.

CARBOHYDRATES

According to Folin and Berglund[1] the sugar of normal urine consists of a motley variety of carbohydrate products and carbohydrate derivatives including di- and polysaccharides. These are believed to arise largely from foreign unusable carbohydrate materials present in grains, vegetables, and fruits, and from decomposition products due to cooking, canning, and baking of food. The ingestion of pure glucose, fructose, maltose, dextrin, or starch does not normally give rise to glycuresis, but the ingestion of impure products or of an ordinary carbohydrate meal increases the sugar of the urine. So also do lactose and galactose. Folin and Benedict have suggested methods for determining, quantitatively, the sugar of normal urine (see Chapter XXXI). Normally sugar is seldom present in amounts sufficient to give the ordinary qualitative tests. During pregnancy and lactation, however, lactose may be found in detectable amounts.

Since sugar is constantly present in urine Benedict[2] has proposed that the term "glycosuria" be discarded and "glycuresis" be used to indicate the presence of an abnormal amount of sugar in the urine. It is believed that 1.5 grams daily is the maximum normal excretion.

Further researches on normal urine sugar have been made by Greenwald, Gross and Samet[3] and by Blatherwick, Bell, Hill and Long.[4]

Folin's Test for Sugar in Normal Urine.[5]—To about 10 c.c. of urine in a test-tube or small flask add about 2 grams of picric acid and about 2 grams of good quality bone-black (Kahlbaum's or Merck's blood charcoal), shake for five minutes, and filter.[6] Add 1 or 2 c.c. of the creatinine-free filtrate to about 10 c.c. of the

[1] Folin and Berglund: *Jour. Biol. Chem.*, 51, 213, 1922.
[2] Benedict, Osterberg and Neuwirth: *Jour. Biol. Chem.*, 34, 217, 1918.
[3] Greenwald, Gross and Samet: *Jour. Biol. Chem.*, 62, 401, 1925.
[4] Blatherwick, Bell, Hill and Long: *Jour. Biol. Chem.*, 66, 801, 1925.
[5] Folin: *Jour. Biol. Chem.*, 22, 327, 1915.
[6] Concentrated urines, which give the most trouble in testing for sugar, contain from 3 to 5 mg. creatinine per c.c. By the above procedure the creatinine content is reduced to practically nothing—at the most a few hundredths mg. per c.c. being left in the filtrate. Bone-black has very strong adsorbing properties for the picrates of creatinine. By allowing the urine and picric acid to stand for a longer time (half an hour or over night) the addition of bone-black may be omitted if desired. The filtrate in that case will contain about 0.1 mg. per c.c., a quantity too small to interfere with the test for sugar.

freshly mixed sugar reagent[1] in a large test-tube (together with a pebble or two to prevent bumping) and boil *with constant shaking*[2] for one and one-half minutes. If the sugar present is considerable (above the normal variations), a typical reduction is obtained. If the trace of sugar is smaller, but still rather large, the whole solution will became turbid as in Benedict's test. If no such turbidity is produced and the boiling mixture remains clear, transfer it at once (*i.e.*, while still very hot) to a centrifuge tube and centrifuge for one or two minutes. Typical red cuprous oxide such as is obtained with pure sugar solutions will be found in the bottom of the centrifuge tube below the green crystalline potassium picrate which usually forms as the liquid cools.

Some copper reagents made as described above give a slight cuprous oxide sediment when boiled alone, *i.e.*, without any added sugar or urine. When that is the case the reagent must be boiled and centrifuged once before using it for the test.

ENZYMES

Various types of enzymes produced within the organism are excreted in both the feces and the urine. In this connection it is interesting to note that *pepsin*, *rennin*, *lipase* and an *amylase* have been positively identified in the urine. The occurrence of trypsin in the urine, at least under normal conditions, is questioned.

VOLATILE FATTY ACIDS

Acetic, butyric, and formic acids have been found under normal conditions in the urine of man and of certain carnivora as well as in the urine of herbivora. Normally they arise principally from the fermentation of carbohydrates and the putrefaction of proteins. The acids containing the fewest carbon atoms (formic and acetic) are found to be present in larger percentage than those which contain a larger number of such atoms. The volatile fatty acids occur in normal urine in traces, the total output for 24 hours according to older investigators varying from 0.008 gram to 0.05 gram.

Pathologically, the excretion of volatile fatty acids is increased in diabetes, fevers, and in certain hepatic diseases in which the parenchyma of the liver is seriously affected. Under other pathological conditions the output may be diminished. These variations, however, in the excretion of the volatile fatty acids possess very little diagnostic value.

[1] Folin's Sugar Reagent.—The reagent is made up in two solutions:

A. Five grams of crystallized copper sulphate are dissolved in 100 c.c. of hot water and to the cooled solution are added 60–70 c.c. of pure glycerol.

B. One hundred and twenty-five grams of anhydrous potassium carbonate are dissolved in 400 c.c. of water.

One part of the glycerol-copper solution (A) is mixed with two parts of potassium carbonate solution (B). Only small portions should be mixed at a time as the reagent (after mixing) does not keep but undergoes gradual reduction.

[2] The shaking is desirable to avoid bumping and is necessary to prevent superheating and consequent reduction of the reagent on the sides of the test tube.

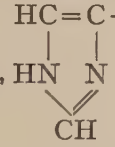
PARALACTIC ACID, $CH_3 \cdot CH(OH) \cdot COOH$.

Paralactic acid is supposed to pass into the urine when the supply of oxygen in the organism is diminished through any cause, *e.g.*, in eclampsia, acute yellow atrophy of the liver, carbon-monoxide poisoning, acute phosphorus poisoning, or epileptic attacks. This acid has also been found in the urine of healthy persons following the physical exercise incident to prolonged marching. Liljestrand and Wilson[1] found the output of lactic acid to vary from 140 mg. to 1370 mg. after a short period (2–3 min.) of strenuous physical exercise (stair running). See experiment, Chap. XXXII. Paralactic acid has been detected in urine of birds after the removal of the liver. Underhill reports the occurrence of this acid in the urine of a case of pernicious vomiting of pregnancy.

PHENACETURIC ACID, $C_6H_5 \cdot CH_2 \cdot CO \cdot NH \cdot CH_2 \cdot COOH$.

Phenaceturic acid occurs principally in the urine of herbivorous animals. It may be isolated from the urine of the dog after feeding phenylacetic acid.[2] It is produced in the organism through the synthesis of glycocoll and phenylacetic acid. It is doubtful if it occurs in normal human urine even after the ingestion of phenylacetic acid. It may be decomposed into its component parts by boiling with dilute mineral acids. The crystalline form of phenaceturic acid (small rhombic plates with rounded angles) resembles one form of uric acid crystal.

UROCANIC ACID,



This acid has been found in the urine of dogs, but not in human urine. It is *imidazolyl-acrylic acid*. Hunter[3] found it among the pancreatic digestion products of casein. It crystallizes as sickle-shaped crystals.

PHOSPHORIZED COMPOUNDS

Phosphorus in organic combination has been found in the urine in such bodies as glycerophosphoric acid, which may arise from the decomposition of lecithin, and phosphocarnic acid. It is claimed that

[1] Liljestrand and Wilson: *Jour. Biol. Chem.*, 65, 773, 1925.
[2] Sherwin: Dissertation, Tübingen, 1915.
[3] Hunter: *Jour. Biol. Chem.*, 11, 537, 1913.

on the average about 2.5 per cent of the total phosphorus elimination is in organic combination.

PIGMENTS

There are at least three pigments normally present in human urine. These pigments are *urochrome*, *urobilin*, and *uroerythrin*.

A. UROCHROME

This is the principal pigment of normal urine and imparts the characteristic yellow color to that fluid. It is apparently closely related to its associated pigment urobilin since the latter may be readily converted into urochrome through evaporation of its aqueous-ether solution. Urochrome may be obtained in the form of a brown, amorphous powder which is readily soluble in water and 95 per cent alcohol. It is less soluble in absolute alcohol, acetone, amyl alcohol and acetic ether, and insoluble in benzene, chloroform, and ether. Urochrome is said to be a nitrogenous body (4.2 per cent nitrogen), free from iron. Urochrome is believed to be identical with the yellow pigment, *lactochrome*, of milk whey.[1] The chromogen of urochrome, *i.e.*, *urochromogen* is present in the urine in pulmonary tuberculosis. Its presence is said to be of prognostic value (see page 673).

B. UROBILIN

Urobilin, which was at one time considered to be the principal pigment of urine, in reality contributes little toward the pigmentation of this fluid. It is claimed that no urobilin is present in freshly voided normal urine but that its precursor, a chromogen called *urobilinogen*, is present and gives rise to urobilin upon decomposition through the influence of light. It is claimed by some investigators that there are various forms of urobilin, *e.g.*, normal, febrile, physiological, and pathological. Urobilin is said to be very similar to, if not absolutely identical with, hydrobilirubin (see page 310). It may be determined quantitatively.[2]

Urobilin may be obtained as an amorphous powder which varies in color from brown to reddish-brown, red and reddish-yellow, depending upon the way in which it is prepared. It is easily soluble in ethyl alcohol, amyl alcohol, and chloroform, and slightly soluble in ether, acetic ether, and in water. Its solutions show characteristic absorption bands (see Absorption Spectra, Plate II). Under normal conditions urobilin is derived from the bile pigments in the intestine.

Urobilin is increased in most acute infectious diseases such as *ery-*

[1] Palmer and Cooledge: *Jour. Biol. Chem.*, 17, 251, 1914.]
Pelkan: *Jour. Biol. Chem.*, 43, 237, 1920.
[2] Marcussen and Hansen: *Jour. Biol. Chem.*, 36, 381, 1918.

sipelas, malaria, pneumonia, and *scarlet fever.* It is also increased in *appendicitis, carcinoma of the liver, catarrhal icterus, pernicious anemia,* in cases of poisoning by antifebrin, antipyrin, pyridin, and potassium chlorate and quite commonly after salvarsan injections. In general it is usually increased when blood destruction is excessive and in disturbances of the liver. It is markedly decreased in phosphorus poisoning.

In liver disease, of any type, *urobilinogen* occurs in the urine. Its detection is the basis of a specific test for *functional liver incapacity.* (See p. 768.)

Experiments

1. Ammoniacal Zinc Chloride Test.—Render some of the urine ammoniacal by the addition of ammonium hydroxide, and after allowing it to stand a short time filter off the precipitate of phosphates and add a few drops of zinc chloride solution to the filtrate. Observe the production of a greenish fluorescence. Examine the fluid by means of the spectroscope and note the absorption band which occupies much the same position as the absorption band of urobilin in acid solution (see Absorption Spectra, Plate II).

2. Ether-Absolute Alcohol Test.—Mix urine and pure ether in equal volumes and shake gently in a separatory funnel. Separate the ether extract, evaporate it to dryness, and dissolve the residue in 2–3 c.c. of absolute alcohol. Note the greenish fluorescence. Examine the solution spectroscopically and observe the characteristic absorption band (see Absorption Spectra, Plate II).

3. Ring Test.—Acidify 25 c.c. of urine with 2–3 drops of concentrated hydrochloric acid, add 5 c.c. of chloroform and shake the mixture. Separate the chloroform, place it in a test-tube, and add carefully 3–5 c.c. of an alcoholic solution of zinc acetate. Observe the formation of a green ring at the zone of contact of the two fluids. If the tube is shaken a fluorescence may be observed.

4. Spectroscopic Examination.—Acidify the urine with hydrochloric acid and allow it to remain exposed to the air for a few moments. By this means if any urobilinogen is present it will be transformed into urobilin. The urine may now be examined by means of the spectroscope. If urobilin is present in the fluid the characteristic absorption band lying between *b* and *F* will be observed (see Absorption Spectra, Plate II). It may be found necessary to dilute the urine with water before a distinct absorption band is observed. This test may be modified by acidifying 10 c.c. of urine with hydrochloric acid and shaking it gently with 5 c.c. of amyl alcohol. The alcoholic extract when examined spectroscopically will show the characteristic urobilin absorption band. (Note the spectroscopic examination in experiment (1) above.)

5. Iodine Test (Gerhardt).—To 20 c.c. of urine add 3–5 c.c. of chloroform and shake well. Separate the chloroform extract and add to it a few drops of iodine solution (I in KI). Render the mixture alkaline with dilute solution of potassium hydroxide and note the production of a yellow or yellowish-brown color. The solution ordinarily exhibits a greenish fluorescence.

6. Alcoholic Zinc Chloride Test (Wirsing).—To 20 c.c. of urine add 3–5 c.c. of chloroform and shake gently. Separate the chloroform extract and add to it a drop of an alcoholic solution of zinc chloride. Note the rose-red color and the

greenish fluorescence. If the solution is turbid it may be rendered clear by the addition of a few cubic centimeters of absolute alcohol.

C. UROERYTHRIN

This pigment is frequently present in small amount in normal urine. The red color of urinary sediments is due in great part to the presence of uroerythrin. It is easily soluble in amyl alcohol, slightly soluble in acetic ether, absolute alcohol, or chloroform, and nearly insoluble in water. Dilute solutions of uroerythrin are pink in color while concentrated solutions are orange red or bright red; none of its solutions fluoresce. Uroerythrin is increased in amount after strenuous physical exercise, digestive disturbances, fevers, certain liver disorders, and in various other pathological conditions.

PURINE BASES

The purine bases found in human urine are adenine, carnine, epiguanine, episarkine, guanine, xanthine, heteroxanthine, hypoxanthine, paraxanthine, and 1-methylxanthine. The main bulk of the purine base content of the urine is made up of *paraxanthine*, *heteroxanthine* and *1-methylxanthine*, which are derived for the most part from the caffeine, theobromine, and theophylline of the food. The total purine base content is made up of the products of two distinct forms of metabolism, *i.e.*, metabolism of ingested nucleins and purines and metabolism of tissue nuclein material. Purine bases resulting from the first form of metabolism are said to be of *exogenous* origin, whereas those resulting from the second form of metabolism are said to be of *endogenous* origin. The daily output of purine bases by the urine is extremely small and varies greatly with the individual (16–60 mg.). The output is increased after the ingestion of nuclein material as well as after the increased destruction of leucocytes. A well-marked increase accompanies leukemia. Edsall and others have shown that the output of purine bases by the urine is increased as a result of X-ray treatment. The purine bases form a higher percentage of the total purine excretion in the case of the monkey, sheep and goat than in man.

Experiment

1. Formation of the Silver Salts.—Add an excess of magnesia mixture[1] to 25 c.c. of urine. Filter off the precipitate and add ammoniacal silver solution[2] to the filtrate. A precipitate composed of the silver salts of the various purine bases is produced. The purine bases may be determined quantitatively by means

[1] Magnesia mixture may be prepared as follows: Dissolve 175 grams of $MgSO_4$ and 350 grams of NH_4Cl in 1400 c.c. of distilled water. Add 700 grams of concentrated NH_4OH, mix very thoroughly and preserve the mixture in a glass-stoppered bottle.

[2] Ammoniacal silver solution may be prepared according to directions given in Appendix.

of Krüger and Schmidt's method (see page 740), or Welker's method (see page 738).

2. Inorganic Physiological Constituents

Ammonia

Next to urea, ammonia is the most important of the nitrogenous end-products of protein metabolism. Ordinarily about 2.5–4.5 per cent of the total nitrogen of the urine is eliminated as ammonia and on the average this would be about 0.7 gram per day. Under normal conditions the ammonia is present in the urine in the form of the *chloride*, *phosphate*, or *sulphate*. This is due to the fact that combinations of this sort are not oxidized in the organism to form urea, but are excreted as such. This explains the increase in the output as ammonia which follows the administration of the ammonium salts of the mineral acids or of the acids themselves. On the other hand, when ammonium acetate and many other ammonium salts of certain organic acids are administered no increase in the output of ammonia occurs since the salt is oxidized and its nitrogen ultimately appears in the urine as urea. Acid-forming foods (see Chapter XXXII) also increase the ammonia output, whereas the administration of alkalies or of base-forming foods decreases the excretion of ammonia.

Experiments[1] indicate that the nitrogen in food protein may in part be replaced by ammonium salts.

Copious water drinking increases the ammonia output. This fact has been interpreted as indicating a stimulation of the gastric secretion.[2]

The acids formed during the process of protein destruction within the body have an influence upon the excretion of ammonia similar to that exerted by acids which have been administered. Therefore a pathological increase in the output of ammonia is observed in such diseases as are accompanied by an increased and imperfect protein metabolism, and especially in diabetes, in which disease acetoacetic acid and β-hydroxybutyric acid are found in the urine in combination with the ammonia.

Folin claims that a pronounced decrease in the extent of protein metabolism, as measured by the total nitrogen in the urine, is frequently accompanied by a decreased elimination of ammonia. The ammonia elimination is therefore probably determined by other factors than the total protein catabolism as such. Furthermore, he believes that a decided decrease in the total nitrogen excretion is always accompanied

[1] Grafe and Schläpfer: *Zeit. physiol. chem.*, 77, 1, 1912, experiments by Abderhalden in same journal.

[2] Wills and Hawk: *Jour. Am. Chem. Soc.*, 36, 158, 1914.

by a *relative increase* in the ammonia-nitrogen, provided the food is of a character yielding an alkaline ash.

According to Nash and Benedict[1] the kidney is the centre of ammonia production in the body. Bliss claims ammonia formation to be a generalized tissue function[2] while Mann and associates[3] believe that the liver does not have any special part in the production of ammonia.

The quantitative determination of ammonia must be made upon the fresh urine, since upon standing the normal urine will undergo ammoniacal fermentation (see page 585).

Experiments

(See Experiment 2 under Phosphates, page 633.)

Sulphates

Sulphur in combination is excreted in two forms in the urine: first, as *unoxidized, loosely combined* or *neutral sulphur*, and second, as *oxidized* or *acid* sulphur. The *unoxidized* or *neutral* sulphur is excreted mainly as a constituent of such bodies as cystine, cysteine, taurine, hydrogen sulphide, ethyl sulphide, thiocyanates, sulphonic acids, oxyproteic acid, alloxyproteic acid, and uroferric acid. The amount of *neutral* sulphur eliminated is in great measure independent of the extent of protein decomposition or of the total sulphur excretion. In this characteristic it is somewhat similar to the excretion of creatinine. The *oxidized* sulphur is eliminated in the form of sulphuric acid, principally as salts of sodium, potassium, calcium, and magnesium; a relatively small amount occurs in the form of *ethereal* sulphuric acid, *i.e.*, sulphuric acid in combination with such *aromatic* bodies as phenol, indole, skatole, cresol, pyrocatechol, and hydroquinol. Sulphuric acid in combination with Na, K, Ca or Mg is sometimes termed *inorganic* or *preformed sulphuric acid*, whereas the ethereal sulphuric acid is sometimes called *conjugate sulphuric acid*. The greater part of the sulphur is eliminated in the oxidized form, but the absolute percentage of sulphur excreted as the preformed, ethereal or loosely combined type depends upon the total quantity of sulphur present; *i.e.*, there is no definite ratio between the three forms of sulphur which will apply under all conditions. The preformed sulphuric acid may be precipitated directly from acidified urine with $BaCl_2$, whereas the ethereal sulphuric acid must undergo a preliminary boiling in the presence of a mineral acid before it can be so precipitated.

[1] Nash and Benedict: *Jour. Biol. Chem.*, 48, 463, 1921; Benedict and Nash, *Ibid.*, 69, 381, 1926.

[2] Bliss: *Jour. Biol. Chem.*, 67, 109, 1926.

[3] Mann: *Jour. Am. Med. Assn.*, 85, 1472, 1925.

The sulphuric acid excreted in the urine arises principally from the oxidation of protein material within the body; a relatively small amount is due to ingested sulphates. Under normal conditions about 2.5 grams of sulphuric acid (SO_3) are eliminated daily, about 75–95 per cent of this being in the form of sulphates. About 90 per cent of this sulphate excretion is in the form of *inorganic* sulphate and 10 per cent as *ethereal* sulphates. Since the sulphuric acid content of the urine has, for the most part, a protein origin and since one of the most important constituents of the protein molecule is nitrogen, it would be reasonable to suppose that a fairly definite ratio might exist between the excretion of these two elements. However, when we appreciate that the percentage content of N and S present in different proteins is subject to rather wide variations, the fixing of a ratio which will express the exact relation existing between these two elements as they appear in the urine as end-products of protein metabolism is practically impossible. It has been suggested that the ratio 5:1 expresses this relation in a general way.

The sulphate ion is excreted with greater difficulty than any other inorganic radical ordinarily present in normal blood.[1] A retention of 3000 per cent of the normal blood value (0.5 to 1.0 mg. of sulphur per 100 c.c. of blood) has been observed. Sulphate ions are not readily absorbed by the tissues even when present in high concentration in the blood.

Pathologically, the excretion of sulphuric acid by the urine is increased in acute fevers and in all other diseases marked by a stimulated metabolism, whereas a decrease in the sulphuric acid excretion is observed in those diseases which are accompanied by a loss of appetite and a diminished metabolic activity.

Experiments

1. Detection of Inorganic Sulphuric Acid.—Place about 10 c.c. of urine in a test-tube, acidify with acetic acid and add some barium chloride solution. A white precipitate of barium sulphate forms.

2. Detection of Ethereal Sulphuric Acid.—Filter off the barium sulphate precipitate formed in the above experiment, add 1 c.c. of hydrochloric acid and a little barium chloride solution to the filtrate and heat the mixture to boiling for 1–2 minutes. Note the appearance of a turbidity due to the presence of sulphuric acid which has been separated from the ethereal sulphates and has combined with the barium of the $BaCl_2$ to form $BaSO_4$.

3. Detection of Unoxidized or Neutral Sulphur.—Place about 10 c.c. of urine in a test-tube, introduce a small piece of zinc, add sufficient hydrochloric acid to cause a gentle evolution of hydrogen, and over the mouth of the tube place a

[1] Denis and Hobson: *Jour. Biol. Chem.*, 55, 183, 1923.

filter paper saturated with lead acetate solution. In a short time the portion of the paper in contact with the vapors within the test-tube becomes blackened due to the formation of lead sulphide. The nascent hydrogen has reacted with the loosely combined or neutral sulphur to form hydrogen sulphide, and this gas coming in contact with the lead acetate paper has caused the production of the black lead sulphide. Sulphur in the form of inorganic or ethereal sulphuric acid does not respond to this test. (For discussion of neutral sulphur compounds see page 615.)

4. Calcium Sulphate Crystals.—Place 10 c.c. of urine in a test-tube, add 10 drops of calcium chloride solution and allow the tube to stand until crystals form. Examine the calcium sulphate crystals under the microscope and compare them with those shown in Fig. 194.

FIG. 194.—CALCIUM SULPHATE. (*Hensel* and *Weil*.)

Chlorides

Next to urea, the chlorides constitute the chief *solid* constituent of the urine. The principal chlorides found in the urine are those of sodium, potassium, ammonium, and magnesium, with sodium chloride predominating. The excretion of chloride is dependent, in great part, upon the nature of the diet, but on the average, the daily output is about 10–15 grams, expressed as sodium chloride. Copious water drinking increases the output of chlorides considerably whereas strenuous physical exercise has the opposite effect.[1] Because of their solubility, chlorides are never found in the urinary sediment.

Since the amount of chlorides excreted in the urine is due primarily to the chloride content of the food ingested, it follows that a decrease in the amount of ingested chloride will likewise cause a decrease in the chloride content of the urine. In cases of actual fasting the chloride content of the urine may be decreased to *a slight trace* which is derived from the body fluids and tissues. Under these conditions, however, an examination of the blood of the fasting subject will show the percentage of chlorides in this fluid to be approximately normal. This forms a very striking example of the care nature takes to maintain the normal composition of the blood. There is a limit to the power of the body to maintain this equilibrium, however, and if the fasting organism be subjected to the influence of diuretics for a time, a point is reached where the normal composition of the blood can no longer be maintained and a gradual decrease in its chloride content occurs which finally results in death. Death is supposed to result not so much because of a lack of

[1] Wilson, Long, Thompson and Thurlow: *Jour. Biol. Chem.*, 65, 755, 1925.

chlorine as from *a deficiency of sodium*. This is shown from the fact that potassium chloride, for instance, cannot replace the sodium chloride of the blood when the latter is decreased in the manner above stated. When this substitution is attempted the potassium salt is excreted at once in the urine, and death follows as above indicated.

Pathologically the excretion of chlorides may be decreased in some fevers, chronic nephritis, croupous pneumonia, diarrhœa, certain stomach disorders, and in acute articular rheumatism. Any condition accompanied by the formation of an exudate (*e.g.*, pneumonia) will cause a diminished chloride output. In convalescence and with resolution of the exudate the chloride excretion rises again.

Experiment

Detection of Chlorides in Urine.—Place about 5 c.c. of urine in a test-tube, render it acid with nitric acid and add a few drops of a solution of silver nitrate. A white precipitate, due to the formation of silver chloride, is produced. This precipitate is soluble in ammonium hydroxide.

Phosphates

Phosphoric acid exists in the urine in two general forms: First, that in combination with the alkali metals, sodium and potassium, and the radical ammonium; second, that in combination with the alkaline earth metals, calcium and magnesium. Phosphates formed through a union of phosphoric acid with the alkali metals are termed *alkaline phosphates*, or phosphates of the alkali metals, whereas phosphates formed through a union of phosphoric acid with the alkaline earth metals are termed *earthy phosphates* or phosphates of the alkaline earth metals.

Three series of salts are formed by phosphoric acid: *Normal*, B_3PO_4,[1] *mono-hydrogen*, B_2HPO_4, and *di-hydrogen*, BH_2PO_4. The di-hydrogen salts are acid in reaction, and it is claimed that about 60 per cent of the total phosphate content of the urine is in the form of this type of salt, and that the acidity of the urine is due in great part to the presence of *sodium di-hydrogen phosphate* (see page 425). Henderson[2] maintains that "determinations of hydrogen ionization in urine and its behavior toward indicators both support the view that in urine there exists a mixture of *mono-* and *di-hydrogen* phosphates of sodium, ammonium and other bases."

In bones the phosphates occur principally in the form of the normal salts of calcium and magnesium. The mono-hydrogen salts as a class are alkaline in reaction to litmus, and it is to the presence of di-sodium

[1] B represents any of the alkali metals or alkaline earth metals.
[2] Henderson: *Am. Jour. Physiol.*, 15, 257, 1906.

hydrogen phosphate, Na_2HPO_4, that the greater part of the alkalinity of the saliva is due.

The excretion of phosphoric acid is extremely variable, but on the average the total output for 24 hours is about 2.5 grams, expressed as P_2O_5. Ordinarily the total output is mainly in the form of phosphates and is distributed between alkaline phosphates and earthy phosphates approximately in the ratio 2:1. The organic phosphorus of the urine constitutes only 1–4 per cent of the total phosphorus content. The greater part of this phosphoric acid arises from the ingested food, either from the preformed phosphates or more especially from the phosphorus in organic combination such as we find it in *phospho-proteins*, *nucleo-proteins* and *lecithins;* the phosphorus-containing tissues of the body also contribute to the total output of this element. Alkaline phosphates ingested with the food have a tendency to increase the phosphoric acid content of the urine to a greater extent than the earthy phosphates so ingested. This is due, in a measure, to the fact that a portion of the earthy phosphates, under certain conditions, may be precipitated in the intestine and excreted in the feces; this is especially to be noted in the case of herbivorous animals. Since the extent to which the phosphates are absorbed in the intestine depends upon the form in which they are present in the food, under ordinary conditions, there can be no absolute relationship between the urinary output of nitrogen and phosphorus. If the diet is constant, however, from day to day, thus allowing of the preparation of both a nitrogen and a phosphorus balance,[1] a definite ratio may be established. In experiments upon dogs which were fed an exclusive meat diet, the ratio of nitrogen to phosphorus, in the urine and feces, was found to be 8.1:1.

It has been demonstrated by recent investigation that the ingestion of *inorganic* phosphorus compounds may give rise to *organic* phosphorus compounds such as lecithin, phosphatides, nucleoproteins and phospho-proteins. This is an instance of an organic substance synthesized from an inorganic substance. The experiments have been made principally on ducks[2] and hens.[3]

Pathologically the excretion of phosphoric acid is increased in such diseases of the bones as diffuse periostosis, osteomalacia, and rickets; according to some investigators, in the early stages of pulmonary tuberculosis, in acute yellow atrophy of the liver, in diseases which are accompanied by an extensive decomposition of nervous tissue, and after

[1] In metabolism experiments, a statement showing the relation existing between the nitrogen content of the food on the one hand and that of the urine and feces on the other, for a definite period, is termed a *nitrogen balance* or a "balance of the income and outgo of nitrogen" (see chapter on Studies in Metabolism, p. 789).

[2] Fingerling: *Biochem. Zeit.*, 38, 448, 1912.

[3] McCollum and Halpin: *Jour. Biol. Chem.*, 11, 47 (Proceedings), 1912

sleep induced by potassium bromide or chloral hydrate (Mendel). It is also increased after copious water drinking. A decrease in the excretion of phosphates is at times noted in febrile affections, such as the acute infectious diseases, in pregnancy, in the period during which the fetal bones are forming, and in diseases of the kidneys, because of non-elimination.

The so-called "phosphaturias" many times represent decreased acidity and not increased phosphate content of the urine. Such conditions are, however, significant as indicating a possible tendency to the formation of phosphatic calculi.

Experiments

1. Formation of "Triple Phosphate."—Place some urine in a beaker, render it alkaline with ammonium hydroxide, add a small amount of magnesium sul-

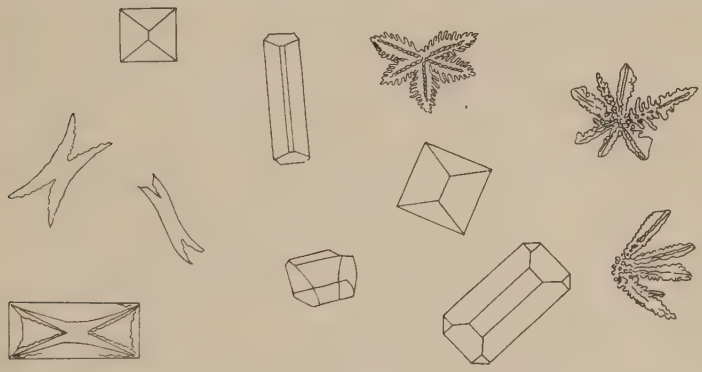


Fig. 195.—"Triple Phosphate." (*Ogden.*)

phate solution and allow the beaker to stand in a cool place over night. Crystals of ammonium magnesium phosphate, "triple phosphate," form under these conditions. Examine the crystalline sediment under the microscope and compare the forms of the crystals with those shown in Fig. 195, above.

2. Ammoniacal Fermentation.—Stand some urine aside in a beaker for several days. Ammoniacal fermentation will develop and "triple phosphate" crystals will form.

(a) Examine the sediment under the microscope and compare the crystals with those shown in Fig. 195.

(b) Hold a glass rod dipped in concentrated hydrochloric acid near the surface of the urine. Note the fumes of ammonium chloride.

(c) Insert a strip of red litmus paper in the urine. Permit the paper to dry. Note the gradual restoration of red color, due to volatilization of ammonia (volatile alkali). Run a control test using 0.5 per cent Na_2CO_3 (fixed alkali).

3. Detection of Earthy Phosphates.—Place 10 c.c. of urine in a test-tube and render it alkaline with ammonium hydroxide. Warm the mixture and note the separation of a precipitate of earthy phosphates.

4. Detection of Alkaline Phosphates.—Filter off the earthy phosphates as formed in the last experiment, and add a small amount of magnesia mixture (see Appendix) to the filtrate. Now warm the mixture and observe the formation of a white precipitate due to the presence of alkaline phosphates. Note the difference in the size of the precipitates of the two forms of phosphates from this same volume of urine. Which form of phosphates was present in the larger amount, earthy or alkaline?

5. Influence upon Fehling's Solution.—Place 2 c.c. of Fehling's solution in a test-tube, dilute it with 4 volumes of water and heat to boiling. Add a solution of sodium dihydrogen phosphate, NaH_2PO_4, a small amount at a time, and heat after each addition. What do you observe? What does this observation force you to conclude regarding the interference of phosphates in the testing of diabetic urine by means of Fehling's test?

Sodium and Potassium

The elements sodium and potassium are always present in the urine. Usually they are combined with such acidic radicals as Cl^-, HCO_3^-, $SO_4^=$ and $HPO_4^=$. The amount of potassium, expressed as K_2O, excreted in 24 hours by an adult, subsisting upon a mixed diet, is on the average 2–3 grams, whereas the amount of sodium, expressed as Na_2O, under the same conditions, is ordinarily 4–6 grams. The ratio of K to Na is generally about 3:5. The absolute quantity of these elements excreted depends, of course, in large measure upon the nature of the diet. Because of the non-ingestion of NaCl and the accompanying destruction of potassium-containing body tissues, the urine during fasting contains more potassium salts than sodium salts.

Pathologically the output of potassium, in its relation to sodium, may be increased during fever; following the crisis, however, the output of this element may be decreased. It may also be increased in conditions associated with acidosis.

Calcium and Magnesium

The greater part of the calcium and magnesium excreted in the urine is in the form of phosphates. The daily output of calcium, which depends principally upon the nature of the diet, aggregates on the average about 0.1–0.4 gram (expressed as CaO) per day. The percentage of calcium salts present in the urine at any one time (10–40 per cent of total calcium output) forms no dependable index as to the absorption of this class of salts, since they are again excreted into the intestine after absorption. It is therefore impossible to draw any satisfactory conclusions regarding the excretion of calcium unless we obtain accurate analytical data from both the feces and the urine.

Myers and Fine[1] have reported data showing a comparison of the kidney and intestine as excretory routes for various inorganic constituents. Their finding in this connection are summarized in the following table:

Number of cases	Moisture content of feces per cent.	Fecal output in per cent of total output of both urine and feces.							
		H_2O	N	S	Cl	P	Ca	Mg	K
5	76	6	10	10	3	36	90	72	18
9	84	16	15	19	9	33	89	68	27

It is not believed that the findings differed especially from the normal, except in that group of cases which suffered from intestinal diarrhea. The average findings in five cases with well formed stools, 74 to 79 per cent. moisture, and those with diarrheal stools, 79 to 89 per cent. moisture have been grouped separately in the table.

Very little is known positively regarding the actual course of the excretion of the calcium under pathological conditions. An excess is found in some diseases of the bones, *e.g.*, osteomalacia. In others as in rickets the urinary excretion may be very low.

The daily excretion of *magnesium* by way of the urine usually amounts to between 0.1 and 0.3 gram, expressed as MgO. The amount depends mainly on the diet. About 50 per cent or more of the excreted magnesium is usually eliminated by the kidneys, the remainder passes out in the feces. There may be a retention of magnesium in certain bone disorders accompanying a loss of calcium; in osteomalacia for example. Thus the excretion of calcium and magnesium do not necessarily run parallel.

Carbonates

Carbonates generally occur in small amount in the urine of man and carnivora under normal conditions, whereas much larger quantities are ordinarily present in the urine of herbivora. The alkaline reaction of the urine of herbivora is dependable in great measure upon the presence of carbonates. In general a urine containing carbonates in appreciable amount is turbid when passed or becomes so shortly after. These bodies ordinarily occur as alkali or alkaline earth compounds and the turbid character of urine containing them is usually

[1] Myers and Fine: *Proc. Soc. Exp. Biol. and Med.*, 16, 73, 1919.

due principally to the latter class of substances. The carbonates of the alkaline earths are often found in amorphous urinary sediments.

Iron

Iron is present in small amount in normal urine. It probably occurs partly in inorganic and partly in organic combination. The iron contained in urinary pigments or chromogens is in organic combination. According to different investigators the iron content of normal urine will probably not average more than 1–5 mg. per day. After splenectomy there is an increased loss of iron from the body particularly by way of the feces (Asher).

Experiment

Detection of Iron in Urine.—Evaporate a convenient volume (10–15 c.c.) of urine to dryness. Incinerate and dissolve the residue in a few drops of iron-free hydrochloric acid and dilute the acid solution with 5 c.c. of water. Divide the acid solution into two parts and make the following tests: (*a*) To the first part add a solution of ammonium thiocyanate; a red color indicates the presence of iron. (*b*) To the second part of the solution add a little potassium ferrocyanide solution; a precipitate of Prussian blue forms upon standing.

Fluorides, Nitrates, Silicates and Hydrogen Peroxide

These substances are all found in traces in human urine under normal conditions. Nitrates are undoubtedly introduced into the organism in the water and ingested food. The average excretion of nitrates is about 0.5 gram per day, the output being the largest upon a vegetable diet and smallest upon a meat diet. Nitrites are found only in urine which is undergoing decomposition and are formed from nitrates in the course of ammoniacal fermentation. Hydrogen peroxide has been detected in the urine, but its presence is believed to possess no pathological importance.

CHAPTER XXVIII

URINE: PATHOLOGICAL CONSTITUENTS[1]

- Glucose.
- Proteins
  - Serum albumin.
  - Serum globulin.
  - Non-blood proteins
    - Proteoses and peptones.
    - Proteins of Bence-Jones, Paton, and Hektoen, Kretschmer and Welker.
  - Nucleoprotein.
  - Fibrin.
  - Oxyhemoglobin.
- Blood
  - Form elements.
  - Pigment.
- Pus.
- Bile.
  - Pigments.
  - Acids.
- Creatine.[2]
- Acetone.
- Acetoacetic acid
- β-Hydroxybutyric acid
- Conjugate glycuronates.
- Pentoses.
- Fat.
- Hematoporphyrin.
- Lactose.
- Galactose.
- Fructose.
- Arsenic.
- Mercury.
- Inositol.
- Melanin.
- Urorosein.
- Urochromogen.
- Unknown substances.

[1] See note at the bottom of page 592.
[2] Normal constituent of urine especially of growing children.

GLUCOSE

Traces of this sugar occur in normal urine,[1] but the amount is not sufficient to be readily detected by the ordinary simple qualitative tests. There are two distinct types of *pathological* glycosuria,[2] *i.e.*, renal glycosuria and diabetes mellitus. In the first type the glycosuria is the result of reduced renal threshold and is not accompanied by hyperglycemia. In diabetes mellitus an increased volume of urine (polyuria) containing sugar is excreted and the blood sugar level is raised. The urine may contain 10 per cent of glucose and the average sugar content is 3–5 per cent. Ordinarily, diabetic urine which contains a high percentage of sugar possesses a faint yellow color, a high specific gravity, and a 24-hour volume which is above normal. Over 100 grams of sugar are daily eliminated in some severe cases of diabetes mellitus.

The official test for glucose adopted by the Association of Life Insurance Medical Directors of America may be found on p. 751.

Experiments

The various tests for glucose in the urine which are embraced in the experiments given herewith are based upon one of the following properties of this sugar:

(1) Its power to *reduce the oxides of certain metals in alkaline solution.*
(2) Its power to *rotate the plane of polarized light.*
(3) Its power to *form crystalline osazones with phenylhydrazine.*
(4) Its ability to *ferment with ordinary yeast.*

1. Phenylhydrazine Reaction.—Test the urine according to one of the following methods: (a) To a small amount of phenylhydrazine mixture (enough to fill the rounded portion of a small test-tube), furnished by the instructor,[3] add 5 c.c. of the urine, shake well, and heat on a boiling water-bath for one-half to three-quarters of an hour. Allow the tube to cool slowly (not under the tap) and examine the crystals microscopically (Plate III, opposite page 72). If the solution has become too concentrated in the boiling process it will be light red in color and no crystals will separate until it is diluted with water.

In case doubtful results are obtained by this test owing to the presence of interfering substances, the urine should be clarified and the test repeated.

[1] Benedict and Osterberg: *Jour. Biol. Chem.*, 55, 769, 1923.
Cole: *Lancet*, 2, 859, 1913;
Folin and Berglund: *Jour. Biol. Chem.*, 51, 213, 1922.
Folin: *Jour. Biol. Chem.*, 22, 327, 1915.
Greenwald, Gross and Samet: *Jour. Biol. Chem.*, 62, 401, 1924.

[2] Inasmuch as urine always contain sugar Benedict, Osterberg and Neuwirth (*Jour. Biol. Chem.*, 34, 217, 1918) suggest that the term "glycuresis" should replace "glycosuria" as indicating an *increased* excretion of sugar.

[3] This mixture is prepared by combining two parts of phenylhydrazine-hydrochloride and three parts of sodium acetate, by *weight*. These are thoroughly mixed in a mortar.

To clarify the urine introduce 10 c.c. into a test-tube, add 1 gram of pure blood charcoal, heat to boiling and allow to stand with occasional shaking for five minutes. Use the filtrate in the test.

Yellow crystalline bodies called *osazones* are formed from certain sugars under these conditions, in general each individual sugar giving rise to an osazone of a definite crystalline form which is typical for that sugar.

It is important to remember in this connection that, of the simple sugars of interest in physiological chemistry, glucose and fructose yield the same osazone, with phenylhydrazine. The melting points of the recrystallized osazones may be used as a further means of identification but since they extend over a range of several degrees and are not far apart for the different sugars, the determination of melting points is of doubtful value. The reaction taking place in the formation of *phenylglucosazone* is shown on p. 72.

In urine particularly it is often difficult to obtain characteristic crystals for sugars other than glucose. Typical crystals of glucosazone are however readily obtained so that the test is a sensitive one for this sugar.

(b) Place 5 c.c. of the urine in a test-tube, add 1 c.c. of phenylhydrazine-acetate solution furnished by the instructor,[1] and heat on a boiling water-bath for one-half to three-quarters of an hour. Allow the liquid to cool slowly and examine the crystals microscopically (Plate III, opposite page 72).

2. Reduction Tests.—To their aldehyde or ketone structure, many sugars owe the property of readily reducing the alkaline solutions of the oxides of metals like copper, bismuth, and mercury; they also possess the property of reducing ammoniacal silver solutions with the separation of metallic silver. Upon this property of reduction the most widely used tests for sugars are based. When whitish-blue cupric hydroxide in suspension in an alkaline liquid is heated it is converted into insoluble black cupric oxide, but if a reducing agent like certain sugars be present the cupric hydroxide is reduced to insoluble yellow or red cuprous oxide. These changes are indicated in formulas given on p. 74.

(a) Fehling's Test.—To about 1 c.c. of Fehling's solution[2] in a test-tube add about 4 c.c. of water, and boil.[3] (The cupric hydroxide is held in solution by the sodium potassium tartrate (Rochelle salt).) This is done to determine whether

[1] This solution is prepared by mixing one part *by volume*, in each case of glacial acetic acid, one part of water and two parts of phenylhydrazine (the base).

[2] See "Reagents and Solutions."

[3] More dilute Fehling's solution should be used in testing urines containing small amounts of sugar. In case of urines containing a high concentration of sugar it may sometimes be desirable to use a larger volume of Fehling's solution.

the solution will of itself cause the formation of a precipitate of brownish-red cuprous oxide. If such a precipitate forms, the Fehling's solution must not be used. Add urine[1] to the hot Fehling's solution, a few drops at a time, and heat the mixture to boiling after each addition (never add more urine than the original volume of Fehling's solution). The production of yellow or brownish-red cuprous oxide indicates that reduction has taken place. The yellow precipitate is more likely to occur if the urine is added rapidly and in large amount, whereas with a less rapid addition of smaller amounts of urine the brownish-red precipitate is generally formed. The differences in color of the cuprous oxide precipitates under different conditions are apparently due to differences in the size of the particles, the more finely divided precipitates having a yellow color, while the coarser ones are red. In the presence of protective colloidal substances the yellow precipitate is usually formed.[2]

This is a more satisfactory test than Trommer's (see p. 751) but even this test is not entirely reliable when used to detect sugar in the urine. Such bodies as *conjugate glycuronates*, *uric acid*, *nucleoprotein*, and *homogentisic acid*, when present in sufficient amount, may produce a result similar to that produced by sugar. *Phosphates of the alkaline earths* may be precipitated by the alkali of the Fehling's solution and in appearance may be mistaken for the cuprous oxide. Cupric hydroxide may also be reduced to cuprous oxide and this in turn be dissolved by *creatinine*, a normal urinary constituent. This will give the urine under examination a greenish tinge and may obscure the sugar reaction even when a considerable amount of sugar is present. According to Laird[3] even small amounts of creatinine will *retard the reaction velocity* of reducing sugars with Fehling's solution.

Conjugate glycuronates[4] are formed after the ingestion of such substances as chloral hydrate, camphor, menthol, thymol, antipyrin, phenol, etc. The chloral hydrate is excreted in the urine as *trichlorethylglycuronate*, $CCl_3 \cdot CH_2 \cdot OOC[CHOH]_4 \cdot CHO$. This compound reduces Fehling's solution and is *levo*rotatory, whereas glucose also reduces but is *dextro*rotatory. Therefore by means of a polariscopic test we may differentiate between a "chloral urine" and a "sugar urine."

In testing urine preserved by *chloroform* a positive test may be obtained in the absence of sugar. This is due to the fact that the hot alkali produces *formic acid* (a reducing fatty acid) from the chloroform.

[1] In case doubtful results are obtained by this test owing to the presence of interfering substances the urine should be clarified and the test repeated. To clarify the urine introduce 10 c.c. into a test-tube, add 1 gram of pure blood charcoal, heat to boiling and allow to stand with occasional shaking for five minutes. Use the filtrate in the test.

[2] Fischer and Hooker: *Science*, N. S. XLV, 505, 1917.

[3] Laird: *Journ. Path. and Bact.*, 16, 398, 1912.

[4] A method for the quantitative determination of conjugate glycuronates based on copper reduction is discussed on p. 744.

Ammonium salts also interfere with Fehling's test. If present in excess the urine should be made alkaline and boiled in order to decompose the ammonium salts.

(b) **Benedict's Test.**[1]—**Benedict has modified the Fehling solution and has succeeded in obtaining one which does not deteriorate upon long standing.**[2] **The following is the procedure for the detection of glucose in the urine: To 5 c.c. of the reagent in a test-tube add 8 (not more) drops of the urine to be examined. The fluid is then boiled vigorously for from one to two minutes and then allowed to cool spontaneously. (Do not hasten cooling by immersion in cold water.) In the presence of glucose the entire body of the solution will be filled with a colloidal precipitate, which may be red, yellow, or green in color, depending upon the amount of sugar present. In the presence of over 0.2–0.3 per cent of glucose the precipitate will form quickly. If no glucose is present, the solution will either remain perfectly clear, or will show a very faint turbidity, due to precipitated urates.**

Even very small quantities of glucose in urine (0.1 per cent) yield precipitates of surprising bulk with this reagent, and the positive reaction for glucose is the filling of the entire body of the solution with a precipitate, so that the solution becomes opaque. Since *amount* rather than color of the precipitate is made the basis of this test, it may be applied, even for the detection of small quantities of glucose, as readily in artificial light as in daylight. Chloroform does not interfere with this test nor does uric acid or creatinine interfere to such an extent as in the case of Fehling's test.

(c) **Folin-McEllroy Test.**[3]—**To 5 c.c. of the reagent**[4] **in a test tube add 5–8 drops of urine (never add more than 0.5 c.c.) and boil for 1–2 minutes or heat in a beaker of boiling water for 3 minutes. If more than the normal traces of sugar be present the hot solution will be filled with a colloidal (greenish-yellow or reddish) precipitate as in Benedict's test. Because of the sensitiveness of this test, when working with urine only a distinctly positive test obtained with the solution still hot is to be regarded as positive.**

[1] Benedict: *Jour. Biol. Chem.*, 5, 485, 1909: *Jour. Am. Med. Ass'n*, 57, 1193, 1911.

[2] Benedict's solution has the following composition:

Copper sulphate	17.3 gm.
Sodium citrate	173.0 gm.
Sodium carbonate (anhydrous)	100.0 gm
Distilled water to	1000.0 c.c.

With the aid of heat dissolve the sodium citrate and carbonate in about 800 c.c. of water Pour (through a folded filter if necessary) into a glass graduate and make up to 850 c.c. Dissolve the copper sulphate in about 100 c.c. of water. Pour the carbonate-citrate solution into a large beaker or casserole and add the copper sulphate solution slowly, with constant stirring and make up to one liter. The mixed solution is ready for use, and does not deteriorate upon long standing.

[3] Folin and McEllroy: *Jour. Biol. Chem.*, 33, 513, 1918.

[4] Folin-McEllroy Reagent.—Dissolve 100 g. of sodium pyrophosphate, 30 g. of disodium phosphate and 50 g. of dry sodium carbonate in approximately 1 liter of water by the aid of a little heat. Dissolve separately 13 g. of copper sulphate in about 200 c.c. of water. Pour the copper sulphate solution into the phosphate-carbonate solution and shake.

(d) **Haines' Test.**—This is a copper reduction test similar in many respects to the Fehling and Benedict reactions. In Haines' reagent[1] the cupric hydroxide is held in solution by glycerol instead of Rochelle salt as in Fehling's solution.

Perform the test as follows: Introduce about 5 c.c. of Haines' reagent[1] into a test-tube and heat to boiling. If no reduction occurs add 6–8 drops of the urine and again bring to a boil. If glucose is present an abundant yellow or brownish-red cuprous oxide precipitate is thrown down. This test is about as delicate as Fehling's test.

(e) **Bismuth Reduction Test (Nylander).—To 5 c.c. of urine in a test-tube add one-tenth its volume of Nylander's reagent[2] and heat for five minutes in a boiling water-bath.[3] The mixture will darken if reducing sugar is present and upon standing for a few moments a black color will appear.**

This color is due to the precipitation of bismuth. If the test is made on urine containing albumin this must be removed, by boiling and filtering, before applying the test, since with albumin a similar change of color is produced. Glucose when present to the extent of 0.08 per cent may be easily detected by this reaction (Rabe[4] claims that 0.01 per cent may be so detected). Uric acid, creatinine and homogentisic acid which interfere with the Fehling test do not interfere with the Nylander's reaction. It is claimed by Bechold that the bismuth reduction tests give a negative reaction with solutions containing sugar when mercuric chloride or chloroform is present. Other observers[5] have failed to verify the inhibitory action of the mercuric chloride and have shown that the inhibitory influence of chloroform may be overcome by raising the temperature of the urine to the boiling-point for a period of five minutes previous to making the test.

Urines rich in *indican*, *uroerythrin*, *urochrome* or *hematoporphyrin*, as well as urines excreted after the ingestion of large amounts of certain *medicinal substances*, may give a darkening of the Nylander's reagent similar to that of a true sugar reaction. It is a disputed point whether the urine after the administration of urotropin will reduce the Nylander reagent.[6]

[1] Haines reagent may be prepared by dissolving 8.314 grams of copper sulphate in 400 c.c. of water adding 40 c.c. of glycerol and 500 c.c. of 5 per cent potassium hydroxide solution.

[2] Nylander's reagent is prepared by digesting 2 grams of bismuth subnitrate and 4 grams of Rochelle salt in 100 c.c. of a 10 per cent potassium hydroxide solution. The reagent is then cooled and filtered.

[3] Hammarsten suggests that the solution be boiled for 2–5 minutes (according to the sugar content) over a free flame and the tube then permitted to stand five minutes before drawing conclusions.

[4] Rabe: *Apoth. Ztg.*, 29, 554, 1914.

[5] Rehfuss and Hawk: *Jour. Biol. Chem.*, 7, 267, 1910; also Zeidlitz: *Upsala Lakäreforen. Forh.*, N. F., 11, 1906.

[6] Abt: *Archives of Pediatrics*, 24, 275, 1907; also Weitbrecht: *Schweiz. Woch.*, 47, 577, 1909.

According to Rustin and Otto the addition of $PtCl_4$ increases the delicacy of Nylander's reaction. They claim that this procedure causes the sugar to be converted *quantitatively*. No quantitative method has yet been devised, however, based upon this principle.

A positive bismuth reduction test is probably due to the following reactions:

(*a*) $$Bi(OH)_2NO_3 + KOH \rightarrow Bi(OH)_3 + KNO_3.$$

(*b*) $$2Bi(OH)_3 - 3O \rightarrow Bi_2 + 3H_2O.$$

Bohmansson,[1] before testing the urine under examination treats it (10 c.c.) with ⅕ volume of 25 per cent hydrochloric acid and ½ volume of boneblack. This mixture is shaken one minute, then filtered, and the neutralized filtrate tested by Nylander's reaction. Bohmansson claims that this procedure removes certain interfering substances, notably urochrome.

3. Fermentation Test.—Rub up in a mortar about 15 c.c. of the urine with a small piece of compressed yeast. Transfer the mixture to a saccharometer (Fig. 19, page 79) and stand it aside in a warm place for about 12 hours. If glucose is present, alcoholic fermentation will occur and carbon dioxide will collect as a gas in the upper portion of the tube. On the completion of fermentation, introduce, by means of a bent pipette, a little KOH solution into the graduated portion, place the thumb tightly over the opening in the apparatus and invert the saccharometer. Remembering that KOH has the power to absorb CO_2 how do you explain the result?[2]

Mathews[3] suggests an easy method for differentiation and estimation of lactose in presence of glucose, based on reduction before and after fermentation with yeast. Castellani and Taylor[4] claim that ordinary samples of yeast will, as a rule, ferment not only glucose but fructose, maltose, sucrose, galactose and lactose, as well. They suggest the use of a fungus *Monilia balcanica Castellani*, which ferments only glucose.

4. Polariscopic Examination.—For directions as to the use of the polariscope see Chapter III.

PROTEINS

Normal urine contains a trace of protein material, but the amount present is so slight as to escape detection by any of the simple tests in general use for the detection of protein urinary constituents. The following are the more important forms of protein material which have been detected in the urine under pathological conditions:

(1) Serum albumin.

(2) Serum globulin.

[1] Bohmansson: *Biochem. Zeit.*, 19, p. 281.

[2] The findings of Neuberg and associates indicate that the liberation of carbon dioxide by yeast is not necessarily a criterion of the presence of sugar. The presence of an enzyme called carboxylase has been demonstrated in yeast which has the power of splitting off CO_2 from the carboxyl group of amino- and other aliphatic acids.

[3] Mathews: *Jour. Am. Med. Ass'n*, 75, 1568, 1920.

[4] Castellani and Taylor: *Jour. Am. Med. Assn.*, 86, 523, 1926.

(3) Non-blood proteins { Proteoses and peptones. / Proteins of Bence-Jones, Paton, and Hektoen, Kretschmer and Welker.

(4) Nucleoprotein.
(5) Fibrin.
(6) Oxyhemoglobin.

ALBUMIN

Normal urine contains a trace of albumin which is too slight to be detected by the usual procedures.

Albuminuria is a condition in which serum albumin or serum globulin appears in the urine. There are two distinct forms of albuminuria, *i.e.*, *renal* albuminuria and *accidental* albuminuria. Sometimes the terms "true" albuminuria and "false" albuminuria are substituted for those just given. In the renal type the albumin is excreted by the kidneys. This indicates a more serious condition and at the same time is more frequently encountered than the accidental type. Among the causes of renal albuminuria the most common are altered blood pressure and altered kidney structure. In the accidental form of albuminuria the albumin is *not* excreted by the kidneys as in the case in the renal form of the disorder, but arises from the blood, lymph, or some albumin-containing exudate coming into contact with the urine at some point below the kidneys. It has been suggested[1] that albuminurias may be classed as *pre-renal*, *renal* and *post-renal*. The pre-renal type is illustrated by the albuminuria of heart disease, whereas the post-renal form corresponds to what we have called "accidental" albuminuria.

The determination of albumin may be of assistance in following the course of kidney disturbances, but the results can only be interpreted in the light of other clinical findings.

From the work of Welker, Thomas and Hektoen[2] on the crystallization of proteins from nephritic urines it appears that the three serum proteins albumin, euglobulin and pseudoglobulin can combine to form a complex molecule which is crystallizable.

The official test for albumin adopted by the Association of Life Insurance Medical Directors of America may be found on p. 756.

Experiments

(The urine should be filtered before performing these tests.)

Nitric Acid Ring Test (Heller).—Place 5 c.c. of concentrated HNO_3 in a test-tube, incline the tube, and by means of a pipette allow the urine to flow slowly

[1] Bruce: *Lancet*, May 6, 1911, p. 1205.
[2] Welker, Thomas and Hektoen: *J. Am. Med. Assn.*, 86, 1333, 1926.

down the side. The liquids should stratify with the formation of a white zone of precipitated albumin at the point of juncture.

If the albumin is present in very small amount the white zone may not form until the tube has been allowed to stand for several minutes. If the urine is quite concentrated a white zone, due to uric acid or urates, will form upon treatment with nitric acid as indicated. This ring may be easily differentiated from the albumin ring by repeating the test after diluting the urine with 3 or 4 volumes of water, whereupon the ring, if due to uric acid or urates, will not appear. It is ordinarily possible to differentiate between the albumin ring and the uric acid ring without diluting the urine, since the ring, when due to uric acid, has ordinarily a less sharply defined upper border, is generally broader than the albumin ring and frequently is situated in the urine *above* the point of contact with the nitric acid. Concentrated urines also occasionally exhibit the formation, at the point of contact, of a *crystalline* ring with very sharply defined borders. This is urea nitrate and is easily distinguished from the "fluffy" ring of albumin. If there is any difficulty in differentiation a simple dilution of the urine with water, as above described, will remove the difficulty. Various colored zones, due either to the presence of indican, bile pigments, or to the oxidation of other organic urinary constituents, may form in this test under certain conditions. These colored rings should never be confounded with the *white* ring which alone denotes the presence of albumin.

After the administration of certain drugs a white precipitate of *resin acids* may form at the point of contact of the two fluids and may cause the observer to draw wrong conclusions. This ring, if composed of resin acids, will dissolve in alcohol, whereas the albumin ring will not dissolve in this solvent.

Weinberger has shown that a ring closely resembling the albumin ring is often obtained in urines preserved for a considerable time by thymol when subjected to the nitric acid test. The ring is due to the formation of nitrosothymol and possibly nitrothymol. If the thymol is removed from the urine by extraction with petroleum ether[1] previous to adding nitric acid, the ring does not form.

An instrument called the *albumoscope* (*horismascope*) has been devised for use in this test and has met with considerable favor. The method of using the albumoscope is described below.

Use of the Albumoscope.—This instrument is intended to facilitate the making of "ring" tests such as Heller's and Roberts'. In making a test about 5 c.c. of the solution under examination is first intro-

[1] Accomplished readily by gently agitating equal volumes of petroleum ether and the urine under examination for *two minutes* in a test-tube before applying the test.

duced into the apparatus through the larger arm (see Fig. 196), and the reagent used in the particular test is then introduced through the capillary arm and allowed to flow down underneath the solution under examination. If a reasonable amount of care is taken there is no possibility of mixing the two solutions and a definitely defined white "ring" is easily obtained at the zone of contact.

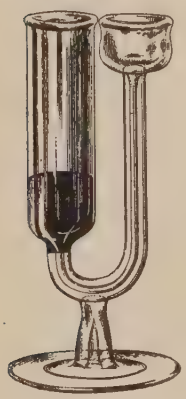


FIG. 196.—ALBUMOSCOPE.

2. Nitric Acid and Magnesium Sulphate Ring Test (Roberts).—Place 5 c.c. of Roberts' reagent[1] in a test-tube, incline the tube, and by means of a pipette allow the urine to flow slowly down the side. The liquids should stratify with the formation of a white zone of precipitated albumin at the point of juncture.

This test is a modification of Heller's ring test and is rather more satisfactory than that test, since the colored rings never form and the consequent confusion is avoided. The *albumoscope* (see Fig. 196) may also be used in making this test.

3. Sulphosalicylic Acid Test (Exton).[2]—To 5 c.c. of urine add an equal volume of Exton's reagent.[3] Mix and warm over a flame. A precipitate denotes the presence of albumin.

4. Coagulation or Boiling Test.—(a) Heat 5 c.c. of urine to boiling in a test-tube. (If the urine is not clear it should be filtered.) A precipitate forming at this point is due either to albumin or to phosphates. Acidify the urine slightly by the addition of 3–5 drops of very dilute acetic acid, adding the acid drop by drop to the hot solution. If the precipitate is due to phosphates it will disappear under these conditions, whereas if it is due to albumin it will not only fail to disappear but will become more flocculent in character, since the reaction of a fluid must be acid to secure the complete precipitation of the albumin by this coagulation process.

Too much acid should be avoided since it will cause the albumin to go into solution. Certain *resin acids* may be precipitated by the acid, but the precipitate due to this cause may be easily differentiated from the albumin precipitate by reason of its solubility in alcohol.

(b) A modification of this test in quite general use is as follows: Fill a test-tube two-thirds full of urine and gently heat the upper half of the fluid to boiling, being careful that this fluid does not mix with the lower half. A turbidity indicates albumin or phosphates. Acidify the urine slightly by the addition of 3–5 drops of dilute acetic acid, when the turbidity, if due to phosphates, will disappear.

[1] Roberts' reagent is composed of 1 volume of concentrated HNO_3 and 5 volumes of a saturated solution of $MgSO_4$.

[2] Exton: *Jour. Am. Med. Assn.*, 80, 529, 1923.

[3] Dissolve 200 grams $Na_2SO_4 \cdot 10H_2O$ in 800 c.c. of water. Cool to 35° and add 50 gms. sulphosalicylic acid. Dissolve and dilute to 1 liter.

Nitric acid is often used in place of acetic acid in these tests. In case nitric acid is used ordinarily 1–2 drops is sufficient.

5. Acetic Acid and Potassium Ferrocyanide Test.—To 5 c.c. of urine in a test-tube add 5–10 drops of acetic acid. Mix well and add potassium ferrocyanide drop by drop, until a precipitate forms.

This is a very delicate test. Schmiedl claims that a precipitate of $K_2ZnFe(CN)_6$ or $Zn_2Fe(CN)_6$ is formed when urines containing zinc are subjected to this test and that this precipitate resembles the precipitate secured with protein solutions. In the case of human urine a reaction was obtained when 0.000022 gram of zinc per cubic centimeter was present. Schmiedl further found that the urine collected from rabbits housed in zinc-lined cages possessed a zinc content which was sufficient to yield a ready response to the test.

Proteoses may also be detected by this test. To differentiate albumin from proteose perform the coagulation test (see above).

GLOBULIN

Serum globulin is not a constituent of normal urine but frequently occurs in the urine under pathological conditions and is ordinarily associated with serum albumin. In albuminuria globulin in varying amounts often accompanies the albumin, and the clinical significance of the two is very similar. Under certain conditions globulin may occur in the urine unaccompanied by albumin.

Experiments

Globulin will respond to all the tests just outlined under Albumin. If it is desirable to differentiate between albumin and globulin in any urine the following process may be employed:

Half-saturation with Ammonium Sulphate.—Place 25 c.c. of neutral urine in a small beaker and add an equal volume of a saturated solution of ammonium sulphate. Globulin, if present, will be precipitated. If no precipitate forms add ammonium sulphate *in substance* to the point of saturation. If albumin is present it will be precipitated upon saturation of the solution as just indicated. This method may also be used to separate globulin and albumin when they occur in the same urine.

Frequently in urine which contains a large amount of urates a precipitate of ammonium urate may occur when the ammonium sulphate solution is added to the urine. This urate precipitate should not be confounded with the precipitate due to globulin. The two precipitates may be differentiated by means of the fact that the urate precipitate ordinarily appears only after the lapse of several minutes whereas the globulin generally precipitates at once.

Howe[1] has recommended the use of sodium sulphate as a precipitant for the different forms of globulin.

[1] Howe: *Jour. Biol. Chem.*, 49, 93, 1921.

NON-BLOOD PROTEINS

Certain proteins are occasionally excreted by the kidneys which do not give the precipitin reactions for any of the normal blood proteins. These include proteoses, peptones, Bence-Jones protein, the Paton protein, and the protein of Hektoen, Kretschmer and Welker.

Proteoses or substances giving similar precipitation reactions have frequently been found in the urine in cases of pneumonia, diphtheria, intestinal ulcer, carcinoma, dermatitis, osteomalacia, atrophy of the kidneys and in conditions in which there is absorption of partially

FIG. 197.—CRYSTALS OF BENCE-JONES PROTEIN.
a. Globules, b. Spicules, c. Needles. (*Hektoen and Welker: J. Inf. Dis.*, 34, 440, 1924.)

digested pus. Peptone occurs much less frequently if at all. In the many instances of peptonuria cited in the early literature the protein may have been proteose.

Bence-Jones protein is believed to be of diagnostic importance in cases of multiple myeloma and myelogenic osteosarcoma. It has been shown to be immunologically distinct from any of the blood proteins,[1] and has been obtained in crystalline form. (See Fig. 197, above.) The protein of Hektoen, Kretschmer and Welker resembles the Bence-Jones and certain proteoses in solubility and precipitation limits with ammonium sulphate (40–60 per cent). It is distinct from these, from

[1] Bayne-Jones and Wilson: *Bull. Johns Hopkins Hosp.*, 33, 37 and 119, 1922; Wilson: *Jour. Biol. Chem.*, 56, 203, 1923; Hektoen and Welker: *J. Infect. Dis.*, 34, 440, 1924.

the Paton protein and from blood proteins in its precipitin reactions, its behavior with heat and in crystalline form.[1] (See Fig. 198, below.)

The protein crystallized by Paton possesses globulin characteristics.[2] In precipitin reactions it is distinct from blood proteins and from the other proteins mentioned.[1,3]

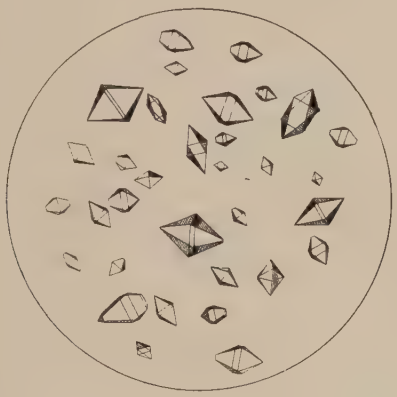


FIG. 198.—PROTEIN OF HEKTOEN, KRETSCHMER AND WELKER.

EXPERIMENTS

1. Phosphotungstic Precipitation Test (v. Aldor).—Acidify 10 c.c. of urine with hydrochloric acid, add phosphotungstic acid until no more precipitate forms and centrifugate[4] the solution. Decant the supernatant fluid, add some absolute alcohol to the precipitate, and centrifugate again. This washing with alcohol is intended to remove the urobilin and hence should be continued so long as the alcohol exhibits any coloration whatever. Now suspend the precipitate in water and add potassium hydroxide to bring it into solution. At this point the solution may be blue in color, in which case decolorization may be secured by gently heating. Apply the biuret test to the cool solution. A positive biuret test indicates the presence of proteoses.

2. Boiling Test.—Make the ordinary coagulation test according to the direction given under Albumin, page 646. If no coagulable protein is found allow the boiled urine to stand and note the gradual appearance, in the cooled fluid, of a flaky precipitate of proteose.

3. Schulte's Method.—Acidify 50 c.c. of urine with dilute acetic acid and filter off any precipitate of nucleoprotein which may form. Now test a few cubic centimeters of the urine for coagulable protein, by tests 2 and 4 under Albumin, page 646. If coagulable protein is present remove it by coagulation and filtration before proceeding. Introduce 25 c.c. of the urine, freed from coagulable protein,

[1] Hektoen, Kretschmer and Welker: *J. Am. Med. Assn.*, 83, 1154, 1924.

[2] Paton: *Proc. Roy. Soc. Edinburgh*, 19, 102, 1892; Bramwell, Byram and Paton: *Rep. Lab. Roy. Coll. Phys. Edinburgh*, 4, 47, 1892; Huppert; *Centr. d. med. Wissensch.*, 36, 481, 1898.

[3] Everett, Bayne-Jones and Wilson: *Bull. Johns Hopkins Hosp.*, 34, 385, 1923.

[4] If not convenient to use a centrifuge the precipitate may be filtered off and washed on the filter paper with alcohol.

into 150 c.c. of absolute alcohol and allow it to stand for 12–24 hours. Decant the supernatant fluid and dissolve the precipitate in a small amount of hot water. Now filter this solution, and after testing again for nucleoprotein with *very dilute* acetic acid, try the biuret test. If this test is positive the presence of proteose is indicated.[1]

Urobilin does not ordinarily interfere with this test since it is almost entirely dissolved by the absolute alcohol when the proteose is precipitated.

4. **Detection of Bence-Jones' Protein.**—Heat the suspected urine very gently, carefully noting the temperature. At as low a temperature as 40°C. a turbidity may be observed, and as the temperature is raised to about 60°C. a flocculent precipitate forms and clings to the sides of the test-tube. If the urine is now acidified *very slightly* with acetic acid and the temperature further raised to 100°C. the precipitate at least partly disappears. Filter while boiling hot. The precipitate returns on cooling the tube.

This property of precipitating at so low a temperature and of dissolving at a higher temperature is typical of "Bence-Jones' protein" and may be used to differentiate it from all other forms of protein material occurring in the urine.

NUCLEOPROTEIN

There has been considerable controversy as to the proper classification for the protein body which forms the "nubecula" of normal urine. By different investigators it has been called *mucin*, *mucoid*, *phosphoprotein*, *nucleoalbumin*, and *nucleoprotein*. Of course, according to the modern acceptation of the meanings of these terms they cannot be synonymous. Mucin and mucoid are glycoproteins and hence contain no phosphorus (see page 178), whereas phosphoproteins and nucleoproteins are phosphorized bodies. It may possibly be that both these forms of protein, *i.e.*, the glycoprotein and the phosphorized type, occur in the urine under certain conditions (see page 620). In this connection we will use the term *nucleoprotein*. The pathological conditions under which the content of nucleoprotein is increased includes all affections of the urinary passages and in particular pyelitis, nephritis, and inflammation of the bladder.

Experiments

1. Detection of Nucleoprotein.—Place 10 c.c. of urine in a small beaker, dilute it with three volumes of water to prevent precipitation of urates, and make the reaction very strongly acid with acetic acid. If the urine becomes turbid it is an indication that nucleoprotein is present.

If the urine under examination contains albumin the greater portion of this substance should be removed by boiling the urine before testing it for the presence of nucleoprotein.

2. Tannic Acid Precipitation Test (Ott).—Mix 25 c.c. of the urine with an equal volume of a saturated solution of sodium chloride and slowly add

[1] If it is considered desirable to test for peptone the proteose may be removed by saturation with $[NH_4]_2SO_4$ according to the directions given on p. 184 and the filtrate tested for peptone by the biuret test.

Almén's reagent.[1] **In the presence of nucleoprotein a voluminous precipitate forms.**

BLOOD

The pathological conditions in which blood occurs in the urine may be classified under the two divisions *hematuria* and *hemoglobinuria*. In hematuria we are able to detect not only the hemoglobin but the unruptured corpuscles as well, whereas in hemoglobinuria the pigment alone is present. Hematuria is brought about through blood passing into the urine because of some lesion of the kidney or of the urinary tract below the kidney. Hemoglobinuria is brought about through hemolysis, *i.e.*, the rupturing of the stroma of the erythrocyte and the liberation of the hemoglobin. This may occur in scurvy, typhus, pyemia, purpura, and in other diseases. It may also occur as the result of a burn covering a considerable area of the body, or may be brought about through the action of certain poisons or by the injection of various substances having the power of dissolving the erythrocytes. Transfusion of blood may also cause hemoglobinuria.

Even in true hematuria the erythrocytes may escape detection if the urine is ammoniacal inasmuch as the cells disintegrate under these conditions.

Experiments

1. Benzidine Reaction.—This is one of the most delicate of the reactions for the detection of blood. Different benzidine preparations vary greatly in their sensitiveness, however. Inasmuch as benzidine solutions change readily upon contact with light, it is essential that they be kept in a dark place.

The test is performed as follows: To 3 c.c. of a saturated solution of benzidine in glacial acetic acid[2] add 2 c.c. of urine and 1 c.c. of 3 per cent hydrogen peroxide. A blue or green color indicates a positive test.

Confirmatory Test.—To 10 c.c. of urine add 1–2 drops of glacial acetic acid and extract by shaking with 5 c.c. of ether. Pour the ether extract into a small evaporating dish. Put on a hot water bath (with flame turned out) and evaporate to dryness. To the residue add a few drops of water, a drop of benzidine solution, and a drop of hydrogen peroxide. A blue or green color indicates blood.

Often when urines containing a small amount of blood are tested by this reaction, the mixture is rendered so turbid as to make it difficult to decide as to the presence of a faint green color. The sensitiveness

[1] Dissolve 5 grams of tannic acid in 240 c.c. of 50 per cent alcohol and add 10 c.c. of 25 per cent acetic acid.

[2] Glacial acetic acid is preferable, but if it is not available, alcohol acidified with acetic acid may be used.

of the benzidine reaction is greater when applied to aqueous solutions than when applied to the urine.

For a modification of this test and further discussion see Chapter XVII on Blood and Lymph.

2. Guaiac Test.—Place 5 c.c. of urine[1] in a test-tube and by means of a pipette introduce a freshly prepared alcoholic solution of guaiac (strength about 1:60) or the Lyle-Curtman[2] guaiac reagent (see p. 321) into the fluid until a turbidity results, then add old turpentine or hydrogen peroxide, drop by drop, until a blue color is obtained.

This is a very delicate test when properly performed. Buckmaster has suggested the use of guaiaconic acid instead of the solution of guaiac. The test is positive both before and after boiling the blood for 15–20 seconds. Pus does not respond after boiling. *Old*, partly putrefied pus gives the test even without the addition of hydrogen peroxide or old turpentine whereas *fresh* pus responds upon the addition of hydrogen peroxide. See discussion on page 343 and test on page 320.

3. Ortho-Tolidin Test (Ruttan and Hardisty).[3]—To 1 c.c. of a 4 per cent glacial acetic acid solution of o-tolidin[4] in a test-tube add 1 c.c. of the solution under examination and 1 c.c. of 3 per cent hydrogen peroxide. In the presence of blood a bluish color develops (sometimes rather slowly) and persists for sometime (several hours in some instances).

This test is said to be as sensitive for the detection of occult blood in feces and stomach contents as is the benzidine reaction. It is also claimed to be more satisfactory for urine than any other blood test. The acetic acid solution may be kept for one month with no reduction in delicacy.

4. Spectroscopic Examination.—Submit the urine to a spectroscopic examination according to the directions given on page 414, looking especially for the absorption bands of oxyhemoglobin and methemoglobin (see Absorption Spectra, Plate I).

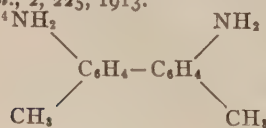
5. Potassium Hydroxide Test (Heller).—Render 10 c.c. of urine strongly alkaline with potassium hydroxide solution and heat to boiling. Upon allowing the heated urine to stand a precipitate of phosphates, colored red by the contained hematin, is formed. It is ordinarily well to make a "control" experiment using normal urine, before coming to a final decision.

Certain substances, such as cascara sagrada, rhubarb, santonin, and senna, cause the urine to give a similar reaction. Reactions due to such substances

[1] Alkaline urine should be made slightly acid with acetic acid as the blue end-reaction is very sensitive to alkali.

[2] Lyle and Curtman: *Jour. Biol. Chem.*, 33, 1, 1918.

[3] Ruttan and Hardisty: *Canadian Medical Assn. Journal*, Nov., 1912, also *Biochemical Bull.*, 2, 225, 1913.

[4]


may be differentiated from the true blood reaction by the fact that both the precipitate and the pigment of the former reaction disappear when treated with acetic acid, whereas if the color is due to hematin the acid will only dissolve the precipitate of phosphates and leave the pigment undissolved.

PUS

Pus may be present in the urine in inflammatory affections of various types. Such a condition is termed *pyuria*. Albumin always accompanies the pus. In catarrh of the bladder and in inflammation of the urethra or of the pelvis of the kidney pus is particularly apt to be present in the urine. If a urine of high pus concentration is voided it may indicate the rupturing of an abscess in some part of the genito-urinary tract. Pus may be detected by one of the procedures given below.

Experiments

1. **Microscopical Detection of Pus.**—The characteristic form elements of pus are leucocytes. They may occur in very small number in normal urine. Examine the urine (centrifugated if necessary) under the microscope. Any considerable number of pus corpuscles indicates a pathological urine. In *acid urine* the pus corpuscles appear as round, colorless cells, composed of refractive, granular protoplasm. Sometimes they may exhibit amœboid movements, particularly if the slide containing them be warmed slightly. They are nucleated (one or more nuclei), the nuclei being clearly visible only upon treating the cells with water, acetic acid or some other suitable reagent. In *alkaline urine* the pus corpuscles are often degenerated. They may occur as swollen, transparent cells, which exhibit no granular structure. If the degeneration has proceeded far enough the nuclei fade and the cell disintegrates and only débris remains

Sometimes it is almost impossible to differentiate between pus corpuscles and certain types of epithelial cells. In such a case apply one of the following chemical tests.

2. **Guaiac Test.**—This test is not specific for pus, but is given by certain other substances and particularly by blood (see Chapter XVII). Perform the test as follows: Acidify the urine (if alkaline) with acetic acid, filter,[1] and add tincture of guaiac or the Lyle-Curtman[2] guaiac reagent (p. 321) to the sediment on the paper. If the pus is old, and partly putrefied it will give a blue color. If no blue color is secured, add old turpentine, or hydrogen peroxide, drop by drop. A blue color formed only under these conditions indicates fresh pus.

As a control test boil some of the urine (or sediment) for 15–20 seconds and repeat the test. Pus does not respond after boiling. In the case of blood the test is positive both before and after boiling.

3. **Potassium Hydroxide Test (Donné).**—Separate the sediment from the urine (by decantation, filtration or centrifugation); place a small piece of solid potassium hydroxide on the sediment and stir. If pus is present (and particularly if it be fresh pus and not disintegrated) the sediment will become slimy and

[1] If desired, the urine may be centrifuged and the sediment used in the test.
[2] Lyle and Curtman: *Jour. Biol. Chem.* 33 1. 1918.

tough. If the sediment is mucus it will more or less pass into solution in the concentrated alkali.

BILE

Both the pigments and the acids of the bile may be detected in the urine under certain pathological conditions. Of the pigments, bilirubin is the only one which has been positively identified in fresh urine; the other pigments, when present, are probably derived from the bilirubin. A urine containing bile may be yellowish-green to brown in color and when shaken foams readily. The staining of the various tissues of the body through the absorption of bile due to occlusion of the bile duct is a prominent symptom of the condition known as icterus or jaundice. Bile is always present in the urine under such conditions unless the amount of bile reaching the tissues is extremely small.

Experiments

Tests for Bile Pigments

Practically all of the tests for bile pigments are based on the oxidation of the pigment by a variety of reagents with the formation of a series of colored derivatives, *e.g.*, biliverdin (green), bilicyanin (blue), choletelin (yellow). However a simple though satisfactory test for bile consists in shaking the urine in a test tube and observing the yellow foam.

1. Gmelin's Test.—To about 5 c.c. of concentrated nitric acid in a test-tube add an equal volume of urine carefully so that the two fluids do not mix. At the point of contact note the various colored rings; green, blue, violet, red, and reddish-yellow.

2. Rosenbach's Modification of Gmelin's Test.—Filter 5 c.c. of urine through a small filter paper. Introduce a drop of concentrated nitric acid into the cone of the paper and observe the succession of colors as given in Gmelin's test.

3. Huppert-Cole Test.[1]—Boil about 15 c.c. of the fluid in a test tube. Add two drops of a saturated solution of magnesium sulphate, then add a 10 per cent solution of barium chloride, drop by drop, boiling between each addition. Continue to add the barium chloride until no further precipitate is obtained. Allow the tube to stand for a minute. Pour off the supernatant fluid as cleanly as possible or use a centrifuge. To the precipitate add 3 to 5 c.c. of 97 per cent alcohol, two drops of strong sulphuric acid, and two drops of a 5 per cent aqueous solution of potassium chlorate. Boil for half a minute and allow the barium sulphate to settle. The presence of bile pigments is indicated by the alcohol solution being colored a greenish blue.

Notes.—To render the test more delicate, pour off the alcoholic solution from the barium sulphate into a dry tube. Add about one-third its volume of chloroform and mix. To the solution add about an equal volume of water, place the thumb on the tube, invert once or twice and allow the chloroform to separate. It contains the bluish pigment in solution.

[1] Cole's "Practical Physiological Chemistry" 7th Edition, pp. 323 and 366, 1926.

The bile pigment is absorbed on to the barium sulphate precipitate, but passes into solution again in acid alcohol. The chlorate acts as a very weak oxidizing reagent, converting bilirubin and biliverdin to the characteristic blue compound.

The author claims that it is a very much more delicate test than Gmelin's Test.

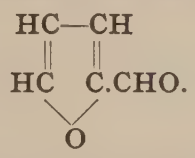
4. Kapsinow's Test.[1]—To 5 c.c. of urine add 0.5 c.c. of Obermayers reagent.[2] Mix and warm if necessary. A green color (biliverdin) shows the presence of bile pigment.

Tests for Bile Acids[3]

1. Sucrose—H_2SO_4 Test (Pettenkofer).—To 5 c.c. of urine in a test-tube add 5 drops of a 5 per cent solution of sucrose. Now incline the tube, run about 2–3 c.c. of concentrated sulphuric acid carefully down the side and note the red ring at the point of contact. Upon slightly agitating the contents of the tube the whole solution gradually assumes a reddish color. As the tube becomes warm, it should be cooled in running water in order that the temperature may not rise about 70°C.

It is claimed that this test is not satisfactory in the presence of protein and chromogenic substances which yield interfering colors with sulphuric acid.

2. Furfural—H_2SO_4 Test (Mylius).—To approximately 5 c.c. of urine in a test-tube add 3 drops of a very dilute (1 : 1000) aqueous solution of furfural,



Now incline the tube, run about 2–3 c.c. of concentrated sulphuric acid carefully down the side and note the red ring as above. In this case also, upon shaking the tube, the whole solution is colored red. Keep the temperature below 70°C. as before.

3. Foam Test (v. Udránsky).—To 5 c.c. of urine in a test-tube add 3–4 drops of a very dilute (1 : 1000) aqueous solution of furfural. Place the thumb over the top of the tube and shake until a thick foam is formed. By means of a small pipette add 2–3 drops of concentrated sulphuric acid to the foam and observe the dark pink coloration produced.

4. Surface Tension Test (Hay).—This test is based upon the principle that bile acids have the property of reducing the surface tension of fluids in which they are contained. The test is performed as follows: Cool about 10 c.c. of urine in a test-tube to 17°C. or lower, and sprinkle a little finely pulverized sulphur upon the surface of the fluid. The presence of bile acids is indicated if the sulphur sinks to the bottom of the liquid, the rapidity with which the sulphur sinks depending upon the amount of bile acids present in the urine. The test is said to react with bile acids when the latter are present in the proportion 1 : 120,000. Allen[4] has suggested the quantitative determination of bile acids by a surface

[1] Kapsinow: *Jour. Am. Med. Assn.*, 82, 687, 1924.

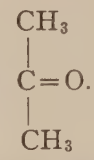
[2] See "Reagents and Solutions."

[3] A *quantitative method* for the estimation of bile acids in urine is described by Schmidt and Merrill: *Jour. Biol. Chem.*, 58, 601, 1923.

[4] Allen: *Jour. Biol. Chem.*, 22, 505, 1915.

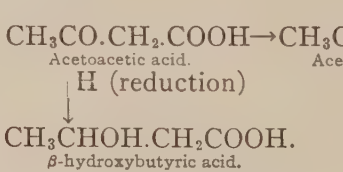
tension method. Urines preserved with thymol may respond positively to this test.

ACETONE,



It was formerly very generally believed that acetone appeared in the urine under pathological conditions because of increased protein decomposition. It is now generally thought that, in man, the output of acetone arises principally from the breaking down of fatty tissues or fatty foods within the organism. The quantity of acetone eliminated has been shown to increase when the subject is fed an abundance of fat-containing food as well as during fasting, whereas a replacement of the fat with carbohydrates is followed by a marked decrease in the acetone excretion. If no carbohydrate food is fed the output of acetone bodies increases at once, producing a *physiological acidosis* (*ketosis*) (see Chapter XXXII on Studies in Metabolism).

Acetone and the closely related bodies, β-hydroxybutyric acid and acetoacetic acid, are generally classified as the *acetone bodies*. They are all associated with a deranged metabolic function and may appear in the urine together or separately, depending upon the conditions. Acetone and diacetic acid may occur alone in the urine but β-hydroxybutyric acid is never found except in conjunction with one or the other of these bodies. Both acetone and β-hydroxybutyric acid may be formed from acetoacetic acid. The relation existing between these three bodies is as follows:[1]



Acetone, chemically considered, is a ketone, *di-methyl ketone*. When pure it is a liquid which possesses a characteristic aromatic fruit-like odor, boils at 56–57°C. and is miscible with water, alcohol, or ether in all proportions. Acetone is a *physiological* as well as a pathological constituent of the urine and under normal conditions the daily output (preformed acetone + acetoacetic acid) is about 3–15 mg.

[1] Maase: *Med. Klinik*, 61, 445, 1910.
Blum: *Münch. med. Woch.*, 57, 683, 1910.
Dakin: *Jour. Biol. Chem.*, 8, 97, 1910.
Marriott: *Jour. Biol. Chem.*, 18, 241, 1914.
Allen: *Am. Jour. Med. Sci.*, 153, 313, 1917.

Pathologically, the elimination of acetone is often greatly increased and at such times a condition of *acetonuria* is said to exist. Values from 0.02–6 grams or higher have been obtained for preformed acetone plus acetone derived from acetoacetic acid. This pathological acetonuria may accompany diabetes mellitus, scarlet fever, typhoid fever, pneumonia, nephritis, phosphorus poisoning, grave anemias, fasting, and a deranged digestive function; it also frequently accompanies auto-intoxication and chloroform and ether anesthesia. The types of acetonuria most frequently met with are those noted in febrile conditions and in advanced cases of diabetes mellitus. The *blood* in diabetic comas has been found to contain as high as 45 mg. of total acetone (acetone + acetoacetic acid) for 100 c.c. of blood serum.

Experiments

1. Isolation from the Urine.—In order to facilitate the detection of acetone in the urine, the specimen under examination should be distilled and the tests as given below applied to the resulting distillate. If it is not convenient to distil the urine, the tests may be conducted upon the undistilled fluid. To obtain an acetone distillate proceed as follows: Place 100–250 c.c. of urine in a distillation flask or retort and render it acid with acetic acid. Collect about one-third of the original volume of fluid as a distillate, add 5 drops of 10 per cent hydrochloric acid and redistil about one-half of this volume. With this final distillate conduct the tests as given below.

2. Gunning's Iodoform Test.—To about 5 c.c. of the urine or distillate in a test-tube add a few drops of Lugol's solution[1] or ordinary iodine solution (I in KI) and a few drops of dilute NH_4OH to form a black precipitate (nitrogen iodide). Allow the tube to stand (the length of time depending upon the content of acetone in the fluid under examination) and note the formation of a yellowish sediment consisting of iodoform. Examine the sediment under the microscope and compare the form of the crystals with those shown in Fig. 18, page 78.

If the crystals are not well formed recrystallize them from ether and examine again. The crystals of iodoform should not be confounded with those of calcium phosphate (Fig. 170, page 542) which may be formed in this test, particularly if made upon the undistilled urine. This test is preferable to Lieben's test (4) since no substance other than acetone will produce iodoform when treated according to the directions for this test; both alcohol and aldehyde yield iodoform when tested by Lieben's test.

Gunning's test is rather satisfactory for the detection of acetone, and has been used with good results even upon the undistilled urine. Protein material apparently interferes with the reaction, and when present the urine should be distilled and the distillate used.[2] In

[1] Lugol's solution may be prepared by dissolving 4 grams of iodine and 6 grams of potassium iodide in 100 c.c. of distilled water.

[2] Rosenbloom: *Jour. Am. Med. Ass'n*, 59, 445, 1912.

some instances where the amount of acetone present is very small it is necessary to allow the tube to stand 24 hours before making the examination for iodoform crystals. This test serves to detect acetone when present in the ratio 1:100,000.

3. Sodium Nitroprusside Test (Legal).—Introduce about 5 c.c. of the urine or distillate into a test-tube, add a few drops of freshly prepared aqueous solution of sodium nitroprusside and render the mixture alkaline with potassium hydroxide. (Be sure to add the nitroprusside before the solution is rendered alkaline.) A ruby-red color, which may be due to creatinine, a normal urinary constituent, or to acetone, or to acetone and creatinine is produced (see Weyl's test, page 609). Add an excess of acetic acid and if acetone is present the red color will be intensified, whereas in the absence of acetone a yellow color will result. Make a control test upon normal urine to show that this is so.

A similar red color may be produced by paracresol in urines containing no acetone.

Two hypotheses have been proposed to explain the color reaction between acetone and nitroprusside: (1) The formation of a complex ion of ferropentacyanide with the isonitroso compound of the ketone, or (2) the formation of such an ion with the isonitroamine derivative of the ketone.[1]

4. Iodoform Test (Lieben).—Introduce 5 c.c. of the urine or distillate into a test-tube, render it alkaline with potassium hydroxide and add 1–2 c.c. of iodine solution drop by drop. If acetone is present a yellowish precipitate of iodoform will be produced. Identify the iodoform by means of its characteristic odor and its typical crystalline form (see Fig. 18, page 78).

While fully as delicate as Gunning's test (2) this test is not as accurate since, by means of the procedure involved, either alcohol or aldehyde will yield a precipitate of iodoform. This test is especially liable to lead to erroneous deductions when urines from the advanced stages of diabetes are under examination, because of the presence of alcohol formed from the sugar through fermentative processes.[2] If protein is present in the urine to be tested it may prevent the acetone from responding to the above reaction. It is therefore advisable to use the distillate to secure most accurate results.[3] Sobel[4] has suggested a quantitative method for acetone based on Lieben's test.

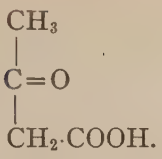
[1] Cambi; *Atti. accad. Lincei*, 22, 376, 1913.

[2] Welker reports the prodution of a pink or red color during the application of this test to the distillates from pathological urines which had been preserved with powdered thymol. He found the color to be due to an idothymol compound which had been previously prepared synthetically by Messinger and Vortmann.

[3] Rosenbloom; *Jour. Am. Med. Ass'n*, 59, 445, 1912.

[4] Sobel; *Schweiz. Apoth. Zig.*, 52, 62, 1914.

ACETOACETIC ACID,



Acetoacetic or diacetic acid occurs in traces in normal urine. The sum of the acetone and the acetoacetic acid excreted in normal urine per day ranges from 3 to 15 mg. and ordinarily three-quarters of this is acetoacetic acid. Under certain pathological conditions it occurs in larger quantities and is rarely found except associated with acetone. In the human body it yields β-hydroxybutyric acid by reduction and upon decomposition yields acetone and carbon dioxide. Acetoaceturia occurs ordinarily under the same conditions as the pathological acetonuria, *i.e.*, in fevers, diabetes, etc. (see Chapter XIX). If very little acetoacetic acid is formed it may be transformed into acetone, whereas if a larger quantity is produced both acetone and acetoacetic acid may be present in the urine. Acetoaceturia is most frequently observed in children, especially accompanying fevers and digestive disorders; it is perhaps less frequently observed in adults, but when present, particularly in fevers and diabetes it is frequently followed by fatal coma.

Acetoacetic acid is a colorless liquid which is miscible with water alcohol and ether, in all proportions. It differs from acetone in giving a violet-red or Bordeaux-red color with a dilute solution of ferric chloride.

Experiments[1]

1. Le Nobel Reaction.[2]—Make 10 c.c. of urine acid with acetic acid, add a few drops of a dilute aqueous solution of sodium nitroprusside and stratify concentrated ammonium hydroxide upon the mixture. In the presence of acetoacetic acid a violet ring forms at once.

Acetone also responds to this test,[3] but the test is more delicate for acetoacetic acid and the response is more prompt.

2. Ferric Chloride Test (Gerhardt).—To 5 c.c. of urine in a test-tube add ferric chloride solution, drop by drop, until no more precipitate forms. In the presence of acetoacetic acid a Bordeaux-red color is produced; this color may be somewhat masked by the precipitate of ferric phosphate, in which case the fluid should be filtered.

A positive result from the above manipulation simply indicates the possible presence of acetoacetic acid. Before making a final decision regarding the presence of this body make the two following control experiments:

[1] To prepare a diacetic acid solution which may be added to urine, if urines containing this acid are not available, proceed as follows: Treat 13 grams of ethyl acetoacetate with 500 c.c. of N/5 sodium hydroxide. Allow to stand for 48 hours to hydrolyze the ester. In preparing urine for tests add 1 part of this solution to 10 parts of urine.

[2] Harding and Ruttan: *Biochem. Jour.*, 6, 445, 1912; also *Biochem. Bull.*, 2, 223, 1913

[3] Bigwood and Ladd: *Jour. Biol. Chem.*, 58, 347, 1923.

(a) Place 5 c.c. of urine in a test-tube, small beaker, or Erlenmeyer flask and boil it vigorously for 3–5 minutes. Cool the vessel and, with the boiled urine, make the test as given above. As has been already stated, acetoacetic acid yields acetone upon decomposition and acetone does not give a Bordeaux-red color with ferric chloride. By boiling as indicated above, therefore, any acetoacetic acid present would be decomposed into acetone and carbon dioxide and the test upon the resulting fluid would be negative. If positive, the color is due to the presence of bodies other than acetoacetic acid.

(b) Place 5 c.c. of urine in a test-tube, acidify with H_2SO_4, to free acetoacetic acid from its salts, and carefully extract the mixture with ether by shaking. If acetoacetic acid is present it will be extracted by the ether. Now remove the ethereal solution, evaporate it to dryness, dissolve the residue in 1–2 c.c. of water and add 3–5 drops of 3 per cent ferric chloride. Acetoacetic acid is indicated by the production of the characteristic Bordeaux-red color.

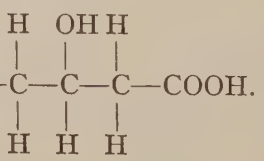
This color disappears spontaneously in 24–48 hours. Such substances as antipyrin, kairin, phenacetin, salicylic acid, salicylates, sodium acetate, thiocyanates, and thallin yield a similar red color under these conditions, but when due to the presence of any of these substances the color does not disappear spontaneously but may remain permanent for days. Many of these disturbing substances are soluble in benzene or chloroform and may be removed from the urine by this means before extracting with ether as above. Acetoacetic acid is insoluble in benzene or chloroform.

Maxwell[1] points out possible error in use of ferric chloride test for acetoacetic acid in urine of patients taking sodium bicarbonate.

3. Sodium Nitrite-Ferrous—Sulphate Reaction (Hurtley).—Place 10 c.c. of urine in a large test-tube, add 2.5 c.c. of concentrated hydrochloric acid and 1 c.c. of fresh 1 per cent sodium nitrite. Shake the tube and permit it to stand for two minutes. Add 15 c.c. of concentrated ammonium hydroxide and 5 c.c. of 10 per cent ferrous sulphate. Shake the tube and permit it to stand. Note the slow development of a violet or purple color in the presence of acetoacetic acid.

This test serves to detect acetoacetic acid when present in a dilution of 1 to 50,000. The concentration of the acetoacetic acid regulates the speed at which the color develops. If the concentration be very low an interval of five hours may elapse before the color appears. The test is believed to be specific for acetoacetic acid.

β-HYDROXYBUTYRIC ACID,



[1] Maxwell: *Med. Jour. Australia*, 1, 458, 1920.

This acid occurs in normal urine in traces, *e.g.*, 20–30 mg. per day.[1] It is found under certain pathological conditions in larger quantities and then always in conjunction with either acetone or acetoacetic acid. It is present in especially large amount in severe cases of diabetes and has also been detected in digestive disturbances, continued fevers, scurvy, measles, and in fasting. It is probable that, in man, β-hydroxybutyric acid, in common with acetone and acetoacetic acid, arises principally from the breaking down of fatty tissues within the organism. Any condition in which large amounts of acetone and acetoacetic acid, and in severe cases β-hydroxybutyric acid also, are excreted in the urine is known as ketonuria. In diabetes the deranged metabolic conditions cause the production of great quantities of these substances which lead to an acid intoxication and ultimately to diabetic coma. In severe diabetes 50-100 grams or over per day may be excreted. In such conditions the β-hydroxybutyric acid may constitute 60-80 per cent of the *total acetone bodies*. In rare cases we may have an excretion of large amounts of β-hydroxybutyric acid with a low acetone output. A ketosis may occur under certain *physiological* conditions (see Chapters XIX and XXXII.)

Ordinarily β-hydroxybutyric acid is an odorless, transparent syrup which is levorotatory and easily soluble in water, alcohol, and ether; it may be obtained in crystalline form.

Experiments

1. Black's Reaction.[2]—Inasmuch as the urinary pigments as well as any contained sugar or acetoacetic acid will interfere with the delicacy of this test when applied to the urine directly, the following preliminary procedure is necessary: Concentrate 10 c.c. of the urine under examination to one-third or one-fourth of its original volume in an evaporating dish at a gentle heat. Acidify the residue with a few drops of concentrated hydrochloric acid, add sufficient plaster of Paris to make a thick paste and allow the mixture to stand until it begins to "set." It should now be stirred and broken up in the dish by means of a stirring rod with a blunt end. Extract the porous meal thus produced twice with ether by stirring and decantation. Any β-hydroxybutyric acid present will be extracted by the ether. Evaporate the ether extract spontaneously or on a water-bath, dissolve the residue in water, and neutralize it with barium carbonate. To 5 to 10 c.c. of this neutral fluid in a test-tube add 2 to 3 drops of ordinary commercial acid hydrogen peroxide. Mix by shaking and add a few drops of Black's reagent.[3] Permit the tube to stand and note the gradual development of a rose color which increases to its maximum intensity and then gradually fades.[4]

[1] Shaffer and Marriott: *Jour. Biol. Chem.*, 16, 265, 1913

[2] Black: *Jour. Biol. Chem.*, 5, 207, 1908.

[3] Made by dissolving 5 grams of ferric chloride and 0.4 gram of ferrous chloride in 100 c.c. of water.

[4] This disappearance of color is due to the further oxidation of the acetoacetic acid.

In carrying out the test care should be taken to see that the solution is *cold* and *approximately neutral* and that a *large excess* of hydrogen peroxide and Black's reagent are not added. In case but little β-hydroxybutyric acid is present the color will fail to appear or will be but transitory if the oxidizing agents are added in *too great excess*. It is preferable to add a few drops of the reagent and at intervals of a few minutes repeat the process until the color undergoes no further increase in intensity. One part of β-hydroxybutyric acid in 10,000 parts of the solution may be detected by this test.

2. **Polariscopic Examination.**—Subject some of the urine (free from protein) to the ordinary fermentation test (see page 79). This will remove glucose and fructose, which would interfere with the polariscopic test. Now examine the fermented fluid in the polariscope and if it is levorotatory the presence of β-hydroxybutyric acid is indicated. This test is not absolutely reliable, however, since conjugate glycuronates are also levorotatory after fermentation.

CONJUGATE GLYCURONATES

Glycuronic acid does not occur free in the urine, but is found, for the most part, in combination with phenol. Much smaller quantities are excreted in combination with indoxyl and skatoxyl. The total content of conjugate glycuronates seldom exceeds 0.004 per cent under normal conditions. The output may be very greatly increased as the result of the administration of antipyrin, borneol, camphor, chloral hydrate, menthol, morphine, naphthol, turpentine, etc. The glycuronates as a group are levorotatory whereas glycuronic acid is dextrorotatory. Most of the glycuronates reduce alkaline metallic oxides and so introduce an error in the examination of urine for sugar. Conjugate glycuronates often occur associated with glucose in glycosuria, diabetes mellitus, and in some other disorders. As a class the glycuronates are non-fermentable. Glycuronic acid has the formula:

$$CHO.(CHOH)_4.COOH.$$

Experiments

1. **Naphthoresorcinol Reaction (Tollens).—Introduce 5 c.c. of urine in a test-tube and add 0.5–1 c.c. of a 1 per cent solution of naphthoresorcinol in 95 per cent alcohol, and 5 c.c. of concentrated hydrochloric acid. Raise the temperature gradually to the boiling-point and boil for one minute, shaking the tube continuously. Stand the tube aside four minutes, then cool under the tap. Extract with an equal volume of ether. Glycuronates are indicated by the ether extract assuming a violet-red color. The spectroscope shows this extract to possess two absorption bands, one on the D line and one to the right of this line.**

2. **Polariscopic-Fermentation Test.—If glucose is present in the urine tested for glycuronates the urine may first be subjected to a polariscopic examination,**

then fermented and a second polariscopic examination made. The sugar being dextrorotatory and fermentable and the glycuronates being levorotatory and non-fermentable the second polariscopic test will show a levorotation indicative of conjugate glycuronates.

3. Reduction-Polariscopic Test.—Test the urine by Benedict's test. If positive try the Resorcinol-HCl reaction for fructose. If negative test the optical activity. Levorotation indicates glycuronates.

PENTOSES

We have two distinct types of pentosuria, *i.e.*, *alimentary pentosuria*, resulting from the ingestion of large quantities of pentose-rich fruits

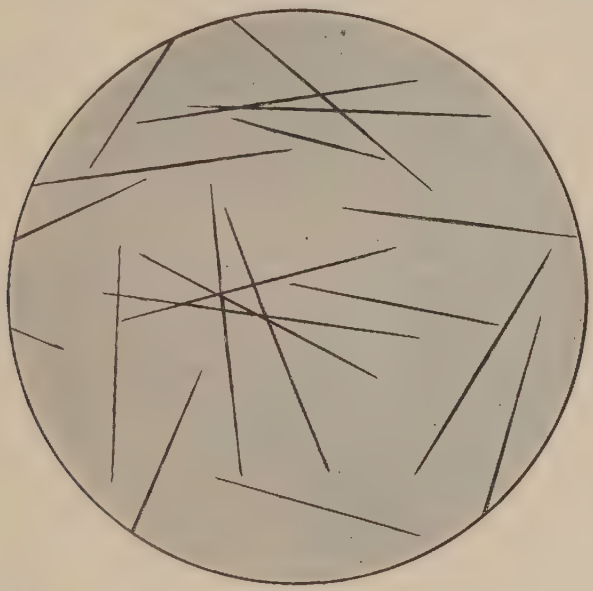


FIG. 199.—PENTOSAZONE CRYSTALS.

Isolated and purified in the author's laboratory by Dr. B. L. Fleming. From the urine of a patient in the service of Dr. S. Solis Cohen, Jefferson Hospital, Phila. For color of crystals see Plate III, opposite page 72.

such as prunes, cherries, grapes, or plums, and fruit juices, in which condition the pentoses appear only *temporarily* in the urine; and the *chronic* form of pentosuria, in which the output of pentoses bears no relation whatever to the quantity and nature of the pentose content of the food eaten. In occurring in these two forms, pentosuria resembles glycosuria (see page 638), but it is definitely known that pentosuria bears no relation to diabetes mellitus and there is no generally accepted theory to account for the occurrence of the chronic form of pentosuria. The pentose detected most frequently in the urine is arabinose, the inactive form generally occurring in chronic pentosuria

although active forms have been more or less frequently reported. Levene and La Forge[1] as well as Zerner and Waltuch[2] report *d*-xylose, and Hiller[3] found *d*-xyloketose in one case of pentosuria. The levorotatory variety occurs in the alimentary type of the disorder. For pentosazone crystals (see Fig. 199).

Experiments

1. Orcinol-Hydrochloric Acid Reaction (Bial).[4]—To 5 c.c. of Bial's reagent[5] in a test-tube add 2–3 c.c. of urine and heat the mixture gently until the first bubbles rise to the surface.[6] Immediately or upon cooling the solution becomes green and a flocculent precipitate of the same color may form.

This test is believed to be more accurate than the original orcinol test. It is claimed that urines containing *menthol*, *kreosotal*, etc., respond to the old orcinol reaction, but not to Bial's. If so desired the osazone of the pentose (see Fig. 199) may be formed, then distilled with hydrochloric acid and the distillate tested by Bial's test (Jolles).

2. Phloroglucinol-Hydrochloric Acid Reaction (Tollens).—To equal volumes of urine and hydrochloric acid (sp. gr. 1.09) add a little phloroglucinol and heat the mixture on a boiling water-bath. Pentose, galactose, or glycuronic acid will be indicated by the appearance of a red color. To differentiate between these bodies examine by the spectroscope and look for the absorption band between D and E given by pentoses and glycuronic acid, and then differentiate between the two latter bodies by the melting-points of their osazones.

FAT

When fat finds its way into the urine through a lesion which brings some portion of the urinary passages into communication with the lymphatic system a condition known as *chyluria* is established. The turbid or milky appearance of such urine is due to its content of chyle. This disease is encountered most frequently in tropical countries, but is not entirely unknown in more temperate climates. Albumin is a constant constituent of the urine in chyluria. Upon shaking a chylous urine with ether the fat is dissolved by the ether and the urine becomes less turbid or entirely clear.

[1] Levene and La Forge: *Jour. Biol. Chem.*, 18, 319, 1914.
[2] Zerner and Waltuch: *Monatsh. Chem.*, 34, 1639, 1913; 35, 1025, 1914.
[3] Hiller: *Jour. Biol Chem.*, 30, 135, 1917.
[4] Bial: *Deut. med. Woch.*, 28, 252, 1902.
[5] Orcinol..... 1.5 grams.
HCl. (conc.)..... 500 grams.
Ferric chloride (10 per cent)..... 20–30 drops.
[6] The test may also be performed by adding the urine to the *hot* reagent. No further heating should be necessary if pentose is present.

HEMATOPORPHYRIN[1]

Urine containing this body is occasionally met with in various diseases, but more frequently after the use of quinine, tetronal, trional, and especially sulphonal. Such urines ordinarily possess a reddish tint, the depth of color varying greatly under different conditions.

Experiments

1. Spectroscopic Examination.—To 100 c.c. of urine add about 20 c.c. of a 10 per cent solution of potassium hydroxide or ammonium hydroxide. The precipitate which forms consists principally of earthy phosphates to which the hematoporphyrin adheres and is carried down. Filter off the precipitate, wash it and transfer to a flask and warm with alcohol acidified with hydrochloric acid. By this process the hematoporphyrin is dissolved and on filtering will be found in the filtrate and may be identified by means of the spectroscope (see page 414, and Absorption Spectra, Plate II).

2. Acetic Acid Test.—To 100 c.c. of urine add 5 c.c. of glacial acetic acid and allow the mixture to stand 48 hours. Hematoporphyrin deposits in the form of a precipitate.

LACTOSE

Lactose is rarely found in the urine except as it is excreted by women during pregnancy, during the nursing period, or soon after weaning. It is rather difficult to show the presence of lactose in the urine in a satisfactory manner, since the formation of the characteristic lactosazone is not attended with any great measure of success under these conditions. It is, however, comparatively easy to show that it is not glucose, for, while it responds to reduction tests, it does not ferment with *pure* yeast and does not give a glucosazone. An absolutely conclusive test, of course, is the isolation of the lactose in crystalline form (Fig. 169, page 537) from the urine.

On oxidation with nitric acid lactose and galactose yield *mucic acid*. This test is frequently used in urine examination to differentiate lactose and galactose from other reducing sugars. To differentiate lactose from pentose, since neither ferments, we may apply the Orcinol-HCl test of Bial, see page 664. To show lactose in the presence of glucose the latter may first be removed by fermentation.[2]

Experiments

1. Mucic Acid Test.—Treat 100 c.c. of the urine under examination with 20 c.c.[3] of concentrated nitric acid and evaporate the mixture in a broad, shallow

[1] Schumm: *Zeit. f. physiol. Chem.*, 98, 123, 1916.
Ellinger and Riesser: *Zeit. f. physiol. Chem.*, 98, 1, 1916.
Fischer: *Zeit. f. physiol. Chem.*, 138, 307, 1924.

[2] Mathews: *Jour. Am. Med. Ass'n*, 75, 1568, 1920.

[3] If the specific gravity of the urine is 1020 or over, it is necessary to use 25–35 c.c. of nitric acid. Under these conditions the mixture should be evaporated until the remaining volume is approximately equivalent to that of the nitric acid added.

glass vessel, upon a boiling water-bath until the volume of the solution is only about 20 c.c. At this point the fluid should be clear and a fine white precipitate of mucic acid should separate.

If the percentage of lactose in the urine is low it may be necessary to cool the solution and permit it to stand for some time before the precipitate will form. It is impossible to differentiate between galactose and lactose by means of this test, but the reaction does serve to differentiate these two sugars from all other reducing sugars. A satisfactory differentiation between lactose and galactose in pure solution may be made by means of Barfoed's test, page 77. This test is, however, not suited for urine examination. To differentiate galactose and lactose in urine use the Phloroglucinol-Hydrochloric Acid Reaction of Tollens, see page 664 and below.

2. Compound Test.—Try the Nylander reaction. If positive try the phenylhydrazine test. If negative (the lactosazone is not readily formed in urine) apply the fermentation test. If this test is also negative, differentiate between lactose and pentose by Orcinol-HCl reaction (Bial) and mucic acid tests.

GALACTOSE

Galactose has occasionally been detected in the urine, and in particular in that of nursing infants afflicted with a deranged digestive function. Lactose and galactose may be differentiated from other reducing sugars which may be present in the urine by means of the mucic acid test. This test simply consists in the production of mucic acid through oxidation of the sugar with nitric acid.

Experiments

1. Mucic Acid Test.—Treat 100 c.c. of the urine under examination with 20 c.c.[2] of concentrated nitric acid and evaporate the mixture in a broad, shallow glass vessel, upon a boiling water-bath, until the volume of the solution is only 20 c.c. At this point the fluid should be clear and a fine, white precipitate of mucic acid should separate.

If the percentage of galactose present in the urine is low it may be necessary to cool the solution and permit it to stand for some time before the precipitate will form. It is impossible to differentiate between galactose and lactose by means of this test, but the reaction does serve to differentiate these two sugars from all other reducing sugars. A satisfactory differentiation between galactose and lactose may be made by the phloroglucinol-hydrochloric acid test of Tollens, below.

2. Phloroglucinol-Hydrochloric Acid Reaction (Tollens).—To equal volumes of the urine and hydrochloric acid (sp. gr. 1.09) add a little phloroglucinol and heat the mixture on a boiling water-bath. Galactose, pentose, and glycuronic acid will be indicated by the appearance of a red color. Galactose may be differentiated

from the two latter substances in that its solutions exhibit no absorption bands upon spectroscopical examination.

FRUCTOSE

Diabetic urine frequently possesses the power of rotating the plane of polarized light to the left, thus indicating the presence of a levorotatory substance. The levorotation is sometimes due to the presence of fructose, although not necessarily confined to this carbohydrate, since conjugate glycuronates and β-hydroxybutyric acid, two other levorotatory bodies, are frequently found in the urine of diabetics. Fructose is invariably accompanied by glucose in diabetic urine, but *fructosuria* has been observed as a separate anomaly. When the percentage of sugar, as determined by the titration method, is greater than the percentage indicated by the polariscopic examination, the presence of fructose may be indicated.

Experiments

1. Borchardt's Reaction.—To about 5 c.c. of urine in a test-tube add an equal volume of 25 per cent hydrochloric acid and a few crystals of resorcinol. Heat to boiling and after the production of a red color, cool the tube under running water and transfer to an evaporating dish or beaker. Make the mixture slightly alkaline with solid potassium hydroxide, return it to a test-tube, add 2–3 c.c. of acetic ether, and shake the tube vigorously. In the presence of fructose the acetic ether is colored yellow.

The only urinary constituents which interfere with the test are *nitrites* and *indican* and these interfere only when they are *simultaneously present.* Under these conditions, the urine should be acidified with acetic acid and heated to boiling for one minute to remove the nitrites. In case the indican content is *very large*, it will impart a *blue* color to the acetic ether, thus masking the yellow color due to fructose. When such urines are to be examined, the indican should first be removed by Obermayer's test (see page 612). The chloroform should then be discarded, the acid-urine mixture diluted with one-third its volume of water, and the test applied as described above. The urine of patients who have ingested *santonin* or *rhubarb* responds to the test. The test will serve to detect fructose when present in a dilution of 1 : 2000, *i.e.*, 0.05 per cent.

2. Resorcinol-Hydrochloric Acid Reaction (Seliwanoff).—To 5 c.c. of Seliwanoff's reagent[1] in a test-tube add a few drops of the urine under examination and heat the mixture to boiling. The presence of fructose is indicated by the production of a red color and the separation of a red precipitate. The latter may be dissolved in alcohol to which it will impart a striking red color.

[1] Seliwanoff's reagent may be prepared by dissolving 0.05 gram of resorcinol in 100 c.c. of dilute (1 : 2) hydrochloric acid.

If the boiling be *prolonged* a similar reaction may be obtained with urines containing glucose. This has been explained[1] in the case of glucose as due to the transformation of the glucose into fructose by the catalytic action of the hydrochloric acid. The precautions necessary for a positive test for fructose are as follows: The concentration of the hydrochloric acid must not be more than 12 per cent. The reaction (red color) and the precipitate must be observed after not more than 20–30 seconds of boiling. Glucose must not be present in amounts exceeding 2 per cent. The precipitate must be soluble in alcohol with a bright red color.

3. Phenylhydrazine Test.—Make the test according to directions under Glucose, 3, page 72.

4. Polariscopic Examination.—A simple polariscopic examination, when taken in connection with other ordinary tests, will furnish the requisite data regarding the presence of fructose, provided fructose is not accompanied by other levorotatory substances, such as conjugate glycuronates and β-hydroxybutyric acid.

ARSENIC

When any soluble form of arsenic is introduced into the body in any way, it is quickly absorbed and distributed by the blood and lymph. The absorption is influenced by the quantity and quality of the food in the stomach, and the activity of the circulation of the part in contact with the poison. Some of the absorbed arsenic may be returned to the alimentary canal by way of the bile and gastro-intestinal mucous membrane. After absorption it may be deposited in the liver, kidneys, brain, bone, muscles, and walls of the stomach and intestines. It is eliminated in all of the excretions, but chiefly by the kidneys and through the feces. It does not appear very promptly in the urine but continues to be excreted in the urine over a long period of time, in some cases for several months. The urine may be examined for arsenic by the following methods.

1. Marsh and Marsh-Berzelius Method.—This method has the advantage of serving as a qualitative and quantitative determination, and is a very delicate test; it is, however, long and tedious. The various steps in the analysis are: (1) the destruction of the organic matter in the urine; (2) treatment with sulphuric acid to drive off excess nitric acid and break up nitro-compounds; and (3) application of independent test to the resultant solution. Proceed as follows: The urine, to which is added one-third its volume of nitric acid, is placed in a casserole or evaporating dish and evaporated at 65°–70°C. to a syrupy consistency. The mass is then allowed to cool and 5 c.c. concentrated sulphuric acid added, and gentle heat applied. The heating must be done cautiously, or deflagration takes place and

[1] Koenigsfeld: *Bioch. Zeit.*, 38, 311, 1912.

some of the arsenic is sure to be lost. The mass will liquefy and finally darken, indicating organic matter. Cool and add concentrated nitric acid, 1 c.c., and apply very gentle heat; copious reddish-brown fumes are evolved. Gradually raise the temperature until darkening of the solution occurs, then cool, add 1 c.c. concentrated nitric acid and again apply gentle heat, and repeat the process until the solution fails to darken. Now raise the temperature until white fumes begin to come off At this temperature excess nitric acid will have been removed and all nitro-compounds broken up. The solution at this point is clear and at most a pale straw color. Cool and add a mixture of 10 c.c. concentrated sulphuric acid and 40 c.c. water, and test for arsenic using a Marsh apparatus. The apparatus (see Fig. 200, below) consists of a wide-mouth flask—250 c.c. capacity—fitted with a two-hole stopper. Through one hole is passed the stem of a separatory funnel of 50 to 60 c.c. capacity. Through the other hole a piece of glass tube bent at right angles, which is fitted to a calcium chloride tube, and this in turn to a narrow quartz tube, the distal end of

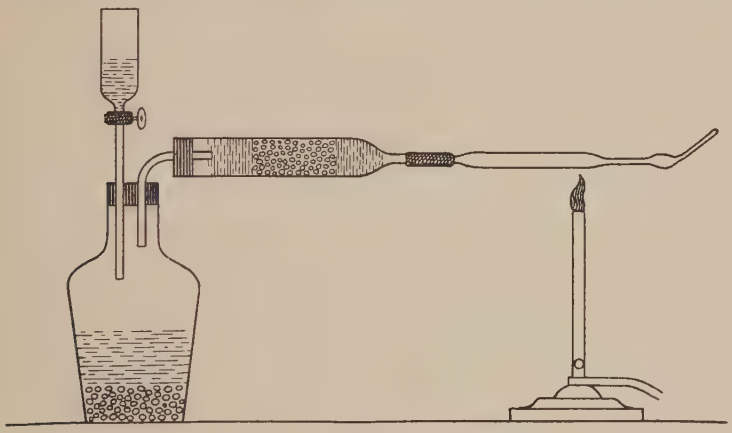


FIG. 200.—MARSH APPARATUS.

which is drawn to a fine bore and bent up almost at a right angle. All joints must be air-tight.

Introduce 30 to 40 grams of arsenic-free granulated zinc into the flask, insert the stopper and through the funnel introduce 50 c.c. dilute sulphuric acid (1 part to 4 parts water). After a few minutes collect a test-tube of gas by inverting a test-tube over the end of the quartz tube, and test it by igniting. When the gas in the test-tube ignites quietly, light the gas issuing from the quartz tube.

Hold a clean porcelain crucible lid in the flame and note whether any deposit occurs. This precaution must be taken to insure that the chemicals and apparatus are not contaminated with arsenic.

Now introduce the prepared urine solution into the funnel and adjust the flow so that 6 to 8 drops are introduced into the flask per minute. Immediately hold a clean porcelain crucible lid in the flame and at the first evidence of a dark deposit apply heat, using a wing-top burner, to the quartz tube. The arsenic if present will deposit in the quartz tube beyond the flame. Now test the spot on the lid to see if it is arsenic; it should dissolve readily in sodium hypochlorite solution. Continue the operation for two hours, remove the Bunsen burner and again hold the lid in the flame. If no more deposits on the lid, the arsenic has all come over

and is deposited in the quartz tube; if deposition occurs, apply the Bunsen burner again and repeat.

When complete, remove the quartz tube, weigh it after cooling, then dissolve out the arsenic with nitric acid, wash, dry, and weigh again. The difference in weight is the weight of metallic arsenic in the volume of urine taken.

2. Reinsch's Test.—This test is very much simpler, but not so delicate. It has the advantage of application in the presence of organic matter. The test is performed as follows: The urine, acidified with one-fifth its volume of pure hydrochloric acid, is placed in a beaker. A piece of bright copper foil free from arsenic is then introduced, and the urine heated almost to the boiling-point. It is then set aside for six to eight hours. The arsenic is deposited on the copper foil, bluish-gray color. The foil is then removed, washed successively in pure water, alcohol, ether, and dried without heat. The foil is then rolled into a scroll and inserted into a 3 mm. bore glass tube 4 inches long, about 1 inch from the end. The tube is then held in the Bunsen flame at an angle of 20 to 25 degrees applying heat where the copper foil is situated. The arsenic volatilizes and is oxidized, and deposits as octahedral crystals of arsenic trioxide on the cooler part of the tube. The crystals can readily be recognized by the microscope and sometimes with a simple magnifying lens.

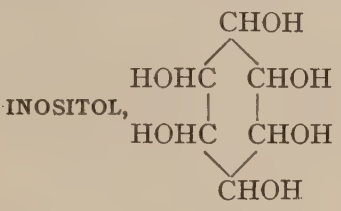
MERCURY

The rapidity of absorption of mercury depends upon a number of conditions such as, mode of administration, the nature of the compound and its physical state, the state and condition of the stomach and intestines, the quantity and quality of the food in the stomach and the state of the circulation of the portal of entrance. There is no definite knowledge as to the form in which it is absorbed. Elimination depends upon the state of the excretory organs. It is eliminated as an albuminate in all the excretions of the body, urine, feces, saliva, sweat, tears, and milk. Elimination begins about two hours after introduction. Depending upon the amount introduced and absorbed, the time required for its complete elimination varies from 24 hours to many weeks. Mercury may be detected in the urine by the following methods.

1. Reinsch's Test.—The procedure is carried out in the same manner as for arsenic (see above). A piece of arsenic-free copper foil is introduced into the urine acidified with one-fifth its volume of pure hydrochloric acid. The urine is, however, not heated to boiling, but warmed to 50° or 60° and set aside for 12 hours or preferably 24 hours. Metallic mercury is deposited on the foil as a bright lustrous mirror. The foil is then washed with pure water, alcohol, ether, and dried without heat, rolled into a scroll, inserted into a glass tube and heated in the same manner as under arsenic. The mercury is deposited in the metallic state in the form of globules readily distinguished with the microscope.

2. Amalgamation Test.—A more rapid method than the above is by amalgamation with zinc. Add 5 grams of zinc dust to the urine and heat for 15 minutes, stirring continuously. Allow the amalgamated zinc to settle and decant the urine.

Then wash by decantation several times with pure water, then with alcohol, and finally with ether and dry in air. Now introduce the dry zinc into a narrow dry glass tube sealed at one end. With the Bunsen burner soften the tube about 2 inches above the zinc and constrict the tube by pulling the ends apart. Introduce a small bit of glass wool or asbestos sufficient to support a small piece of iodine. Introduce the iodine supported by the asbestos at the constriction. Apply heat to the zinc amalgam, and then gently to the region holding the iodine to gently volatilize it, and immediately reapply heat to the zinc. The mercury volatilizes and meeting the iodine vapor unites with it, and is deposited as the red iodide of mercury.



Inositol occasionally occurs in the urine in albuminuria, diabetes mellitus, and diabetes insipidus. It is claimed also that copious water-drinking causes this substance to appear in the urine. Inositol was at one time considered to be a sugar but is now known to be hexahydroxybenzene, as the above formula indicates. It is an example of a non-carbohydrate in whose molecule the H and O are present in the proportion to form water. In other words it has the formula of the hexoses, *i.e.*, $C_6H_{12}O_6$. Inositol occurs widely distributed in the vegetable kingdom, and because of this fact the theory has been voiced that it represents one of the first stages in the conversion of a carbohydrate into the benzene ring. It is found in the liver, spleen, lungs, brain, kidneys, suprarenal capsules, muscles, leucocytes, testes, and urine under normal conditions.

Experiment

1. **Detection of Inositol (Scherer).**—Acidify the urine with concentrated nitric acid and evaporate nearly to dryness. Add a few drops of ammonium hydroxide and a little calcium chloride solution to the moist residue and evaporate the mixture to dryness. In the presence of inositol (0.001 gram) a bright red color is obtained.

For a more satisfactory test, which is also more time-consuming, see Salkowski's[1] modification of Scherer's test.

MELANINS

These pigments never occur normally in the urine, but are present under certain pathological conditions, their presence being especially associated with melanotic tumors. Ordinarily the freshly passed urine

[1] Salkowski: *Zeit. physiol. chem.*, 69, 478, 1910.

is clear, but upon exposure to the air the color deepens and may at last be very dark brown or black in color. The pigment is probably present in the form of a chromogen or melanogen and upon coming into contact with the air oxidation occurs, causing the transformation of the melanogen into melanin and consequently the darkening of the urine.

It is claimed that melanuria is proof of the formation of a visceral melanotic growth. In many instances, without doubt, urines rich in indican have been wrongly taken as diagnostic proof of melanuria. The pigment melanin is sometimes mistaken for indigo and melanogen for indican. It is comparatively easy to differentiate between indigo and melanin through the solubility of the former in chloroform.

In rare cases melanin is found in urinary sediment in the form of fine amorphous granules.

Experiments

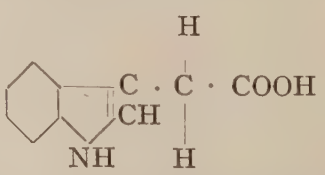
1. Ferric Chloride Reaction (von Jaksch-Pollak).—Add a few drops of ferric chloride solution to 10 c.c. of urine in a test-tube and note the formation of a gray color. Upon the further addition of the chloride a dark precipitate forms, consisting of phosphates and adhering melanin. An excess of ferric chloride causes the precipitate to dissolve.

This is the most satisfactory test for the identification of melanin in the urine.

2. Bromine Test (Zeller).—To 50 c.c. of urine in a small beaker add an equal volume of bromine water. In the presence of melanin a yellow precipitate will form and will gradually darken in color, ultimately becoming black.

UROROSEIN

This is a pigment which is not present in normal urine but may be detected in the urine in various diseases, such as pulmonary tuberculosis, typhoid fever, nephritis, and stomach disorders. Urorosein, in common with various other pigments, does not occur preformed in the urine, but is present in the form of a chromogen, which is transformed into the pigment upon treatment with a mineral acid. Herter[1] showed this chromogen to be indole acetic acid,



Normal urine responds to the urorosein reaction (see below) if nitrites are present.

[1] Herter: *Jour. Biol. Chem.*, 4, 253, 1908.

Experiments

1. Nitrite-Hydrochloric Acid Test (Urorosein Reaction).—To 10 c.c. of urine in a test-tube add 2 c.c. of concentrated hydrochloric acid and a few drops of a 1 per cent solution of potassium nitrite. A rose-red color indicates urorosein.

The chromogen (indole acetic acid) has been changed into urorosein by oxidation.

UROCHROMOGEN

This is the chromogen of urochrome, the normal urinary pigment (see Chapter XXVI). It is claimed that the *urochromogen reaction* of the urine is an aid to prognosis and diagnosis of pulmonary tuberculosis. Urochromogen is not present in normal urine. Its presence in pathological urine is due probably to faulty oxidation, *i.e.*, failure to oxidize the chromogen to urochrome. Urochromogen may be detected by oxidizing it to urochrome by means of potassium permanganate. In this process a certain antecedent of urochromogen is also oxidized to urochrome. Whereas the diazo reaction (see page 674) is also given by urines containing urochromogen, it is claimed that the diazo reaction does not show the presence of the precursor of urochromogen. Hence the urochromogen reaction is said to be more constant and uniform in its appearance.

Experiment

Urochromogen Reaction (Weisz).[1]—Fill a test-tube a little less than one-third full of urine, dilute it with 2 volumes of distilled water and mix thoroughly. Pour one-half the diluted urine into another tube and to one of the tubes add 3 drops of a 1 per cent solution of potassium permanganate. Shake the tube thoroughly. In the presence of urochromogen a yellow tint will appear in the tube to which permanganate was added.

The reaction is due to the oxidation of urochromogen to urochrome, and is believed to be of value as an aid in prognosis and diagnosis of pulmonary tuberculosis. The presence of sugar, albumin or urobilin in low concentration does not interfere with the test. The test often runs parallel with the diazo reaction (see below). The test is supposed to be positive when the focus of the lung is so active or extensive as to flood the blood with toxins or to break down the defensive forces of the body. It is claimed, therefore, that this test will differentiate the cases in which the tuberculosis is beyond help from the tuberculin from those in which the body is liable to respond favorably to its action.[2] Some investigators claim the test is not specific and that a

[1] Weisz: *Münch. med. Woch.*, 58, 1348, 1911.
Heflebower: *Am. Jour. Med. Sci.*, 143, 221, 1912.
Metzger and Watson: *Jour. Am. Med. Ass'n*, 62, 1886, 1914.

[2] M. and A. Weisz: *Wien. klin. Woch.*, 25, 1183, 1912.
Burgess: *Jour. Am. Med. Ass'n*, 66, 82, 1916.

positive reaction will be obtained in many disorders other than tuberculosis.[1]

UNKNOWN SUBSTANCES

1. Ehrlich's Diazo Reaction.—Place equal volumes of urine and Ehrlich's diazobenzenesulphonic acid reagent[2] in a test-tube, mix thoroughly by shaking, and quickly add ammonium hydroxide in excess. The test is positive if both the fluid and the foam assume a red color. If the tube is allowed to stand a precipitate forms, the upper portion of which exhibits a blue, green, greenish-black, or violet color. Normal urine gives a brownish-yellow reaction with the above manipulation.

The exact nature of the substance or substances upon whose presence in the urine this reaction depends is not well understood. The reaction may be taken as a metabolic symptom of certain disorders, which is of value diagnostically *only* when taken in connection with the other symptoms. The reaction appears principally in the urine in febrile disorders and in particular in the urine in typhoid fever, tuberculosis, and measles. The reaction has also been obtained in the urine in various other disorders such as carcinoma, chronic rheumatism, diphtheria, erysipelas, pleurisy, pneumonia, scarlet fever, syphilis, typhus, etc. The administration of alcohol, chrysarobin, creosote, cresol, dionin, guaiacol, heroin, morphine, naphthalene, opium, phenol, tannic acid, etc., will also cause the urine to give a positive reaction.

Hunter[3] describes two types of diazo reaction in alkaline solution—Type A and Type B. Type A reaction is characterized by a *gradual* development of color until a relatively stable maximum is reached. Type B is characterized by an *immediate* development and a very rapid disappearance of color. All urines give some form of Type A reaction and the substances responsible are iminazoles, phenols, purines and

[1] Tuliato: *Gazetta d. Osp. e delle Clin.*, 35, 1914.
Martelli and Pizzetti: *Policlinico*, 21, April 1, 1914.

[2] Two separate solutions should be prepared and mixed in definite proportions when needed for use:
(*a*) Five grams of sodium nitrite dissolved in 1 liter of distilled water.
(*b*) Five grams of sulphanilic acid and 50 c.c. of hydrochloric acid in 1 liter of distilled water.
Solutions *a* and *b* should be preserved in well-stoppered vessels and mixed in the proportion 1:50 when required. Green asserts that greater delicacy is secured by mixing the solutions in the proportion 1:100. The sodium nitrite deteriorates upon standing and becomes unfit for use in the course of a few weeks.
The following chemical reactions take place in preparing the reagent:

(*a*) $NaNO_2 + HCl \rightarrow HNO_2 + NaCl.$

(*b*) $C_6H_4(NH_2)(HSO_3) + HNO_2 \rightarrow C_6H_4{<}{N \atop SO_3}{>}N + 2H_2O.$

Sulphanilic acid — Diazo-benzenesulphonic acid.

[3] Hunter: *Bioch. Jour.*, 19, 25, 1925.

probably unknown chromogens. Type B is given only by pathological urines, especially those from *typhoid* and *measles*. Urochromogen may be responsible for this reaction.[1]

TESTS FOR KIDNEY EFFICIENCY

1. Phenolsulphonephthalein Test.—*Principle.*—This test for renal function was devised by Rowntree and Geraghty.[2] It depends upon the injection into the tissues of a dyestuff which is eliminated rapidly by the normal kidneys, and can be easily estimated quantitatively in the urine.

This dyestuff, phenolsulphonephthalein, is non-irritative to the body either when taken by mouth or when injected into the tissues,[3] so that it does no harm to an already weakened kidney.

The patient upon whom the test is to be performed is given 300–400 c.c. of water 20–30 minutes previously, in order to assure a free flow of urine.

Procedure.—One c.c. of a solution containing 6 mg. of phenolsulphonephthalein[4] per c.c. is injected intramuscularly in the lumbar region, the time of injection being noted. The patient is then catheterized and the urine as it forms thereafter allowed to drop into a beaker containing 2 drops of 25 per cent NaOH. The appearance of a red color in the alkalinized urine indicates beginning excretion of the drug, the normal time being within 5 to 10 minutes after its injection. Urine is now collected in one-hour samples. In patients with obstruction to the flow of urine from the bladder the retention catheter is stoppered and the urine drawn off at the end of each hour. Other patients may simply be allowed to urinate at the hourly periods.

To each hour sample of urine is added 25 per cent NaOH, drop by drop, until the maximum intensity of color appears. This color will remain constant for an indefinite period of time. Each sample is then placed in a 1000 c.c. volumetric flask and diluted to the mark with distilled water.

Comparison is made in a Duboscq or other colorimeter (see p. 358) with a standard consisting of 3 mg. of phenolsulphonephthalein in 1000 c.c. of solution. The cylinder containing the standard may conveniently be placed at the 10 mm. mark.

Calculation.—Since the volume of each urine sample is the same as that of the standard, the percentage elimination of phenolsulphonephthalein in each may be easily calculated as follows:

[1] Weiss: *Biochem. Zeit.*, 112, 61, 1920 and Baudisch: *Jour. Biol. Chem.*, 60, 155, 1924.

[2] Rowntree and Geraghty: *Jour. Pharm. and Exper. Therap.*, 1, 579, 1910: also *Arch. Int. Med.*, March, 1912, p. 284.

[3] Abel and Rowntree: *Jour. Pharm. and Exper. Therap.*, 1, 231, 1910.

[4] This solution is prepared by adding 0.6 gram phenolsulphonephthalein and 0.84 c.c. of 2 N NaOH to enough 0.75 per cent NaCl solution to make 100 c.c. This gives the monosodium or acid salt which is slightly irritant locally when injected. It is necessary to add 2–3 drops more 2 N NaOH which changes the color to a bordeaux red. This preparation is non irritant.

Reading of Urine : Reading of Standard :: 50 : X.

The amount of the drug eliminated normally is 40–60 per cent during the first hour and 20–25 per cent during the second hour, or a total of 60–85 per cent for two hours. The amount of the drug excreted has been found to be independent of the quantity of urine obtained. In case of delayed excretion the collection of hourly samples may be continued until practically all of the drug has been recovered in the urine.

If it is desired to test the function of each kidney separately, ureteral catheterization must be resorted to, the experiment otherwise being performed as above described.

The phenolsulphonephthalein test may be used to indicate the amount of derangement in quantitative functional disturbance of the kidneys, as in chronic interstitial and chronic parenchymatous nephritis or uremia.

It is claimed that the rate of excretion of phenolsulphonephthalein is affected by certain extra-renal factors, namely, the albumin concentration and reaction of the blood.[1]

McLean[2] has suggested another method for studying kidney function which is based upon the relationship between the urea content of the blood and the rate at which the urea is excreted by the kidney. (See p. 678.) It gives similar values to the phenolsulphonephthalein test. It has an advantage in that it enables one to measure kidney function by a study of an actual normal function of the organ, *i.e.*, urea excretion. The method, however, is more or less complex.

2. Mosenthal Test for Kidney Function.[3]—*Principle.*—The patient under examination is placed for a day on a more or less definite diet which should contain a sufficient quantity of protein, salt, fluid, and purine derivatives, *i.e.*, diuretic materials such as are present in an ordinary diet. The urine is collected in six two-hour periods during the day and one twelve-hour night period. These urine specimens are analyzed for volume, specific gravity, total nitrogen, and chlorides.

Procedure.—On the day of the test have the patient empty the bladder at 8 A.M. and start the diet for the day which is selected to contain approximately 13–14 grams of nitrogen, 8–9 grams of salt, 1700–1800 c.c. of fluid, and considerable purine material in meat, soup, tea, and coffee.[4] **No solid food nor**

[1] Grollman: *Jour. Biol. Chem.*, 64, 141, 1925.

[2] McLean: *Jour. Am. Med. Ass'n*, 66, 415, 1916.

[3] Mosenthal: *Boston Med. and Surg. Jour.*, 170, 245, 1914.

[4] A diet suitable to ordinary hospital conditions is given by Kahn: "Functional Diagnosis," p. 260, New York, 1920. It is emphasized that the diet need not be exactly the same as that given since the foods found in the ordinary household contain sufficient diuretic materials for the proper carrying out of the test. In private practice it is only necessary to ask the patient to eat three full meals a day and write down the approximate quantities, as—1 cup of coffee, two slices of toast, two tablespoonfuls of oatmeal, etc.

fluid of any kind must be taken between meals and especial care must be observed that nothing is eaten nor drunk during the night. The meals should start at 8 A.M., 12 Noon, and 5 P.M. respectively.

Collect the urine punctually at the end of every two-hour period until 8 P.M. and place in separate bottles. Collect the night urine from 8 P.M. to 8 A.M. of the following day in another bottle. Measure the volume of each specimen of urine and determine in each case the specific gravity, total nitrogen, and total chlorides.

Interpretation.[1]—The test is of particular value apparently as giving earlier indications of diminished kidney efficiency than is true of other tests used. It is sometimes difficult to interpret the results obtained in terms of renal involvement because of the influence of possible extrarenal factors.[2] In general, however, the normal response is one in which the specific gravity figures vary 10 points or more from the highest to the lowest and the volume of the night urine is 750 c.c. or less. If the percentage of nitrogen and sodium chloride in the night urine or in the highest of any of the day specimens is 1 per cent a normal condition is indicated. Values under 1 per cent, however, may or may not be abnormal.

When kidney function becomes involved the first signs are usually demonstrated in the night urine. The quantity becomes increased and the specific gravity and the nitrogen concentration are lowered. One or all of these changes from the normal may occur. In severe cases of *chronic nephritis* an advanced degree of functional inadequacy of the kidney is indicated by a markedly fixed and low specific gravity, a diminished output of both salt and nitrogen, a tendency to total polyuria and a night urine showing an increased volume, low specific gravity, and low concentration of nitrogen. Such functional pictures are, however, not confined to nephritis. They are found frequently in many other conditions: pyelitis, cystitis, hypertrophied prostate, marked anemia, pyelonephritis, polycystic kidney, and diabetes insipidus. The following table taken from Mosenthal shows the response of a normal individual:

[1] Kahn: *loc. cit.*; Mosenthal: *Arch. Int. Med.*, 22, 770, 1918.
[2] Lyle and Sharlit: *Arch. Int. Med.*, 21, 366, 1918.
Mosenthal and Lewis: *Jour. Am. Med. Ass'n.*, 67, 933, 1916.

Time of day	Urine		Sodium chloride		Nitrogen	
	c.c.	Sp. gr.	Per cent	Grams	Per cent	Grams
8–10	153	1.016	1.32	2.02	0.89	1.26
10–12	156	1.019	1.25	1.95	0.74	1.15
12–2	194	1.012	0.64	1.24	0.59	1.14
2–4	260	1.014	0.77	2.00	0.56	1.46
4–6	114	1.020	0.99	1.13	0.95	1.08
6–8	238	1.010	0.43	1.02	0.52	1.235
Total day	1115	.....	.....	9.36	....	7.32
Night, 8–8	375	1.020	0.63	2.36	1.23	4.61
Total, 24 hours	1490	.....	....	11.72	....	11.93
Intake	1760	.....	....	8.5	....	13.4
Balance	+270	.....	....	−3.22	....	+1.47

3. The Urea Concentration Test of MacLean and deWesselow.[1]—The patient is given a definite amount of urea dissolved in water, one hour samples of urine collected, and the concentration of urea determined.

Procedure.—The patient is asked to empty the bladder, and immediately afterwards he receives by mouth 15 grams of urea dissolved in about 100 c.c. of water. The bladder is emptied one hour and two hours after the urea has been given and the specimens of urine examined for urea content. Thus if urea is given at 10 A.M., a specimen of urine is obtained at 11 A.M. and at 12 A.M. Urea is then determined quantitatively. If either specimen gives a percentage of urea above 2, the kidneys are held to be fairly efficient; the higher the concentration the more effective is the renal function. The reason why two specimens are taken is that in certain patients the urea given by mouth may produce a diuresis which tends to dilute the urine passed during the first hour. In this case the second hour's specimen should be examined. Indeed, in routine work it is generally best to discard the first specimen altogether, and to rely on the result obtained from the second specimen. Not more than about 120 c.c. urine should be passed in the second hour. Occasionally, if there is much available fluid in the patient's system, it may be necessary to take a specimen after three hours, or even to repeat the test, but this is seldom necessary. In patients with marked diuresis this must be allowed for in estimating the renal function.

4. The Amylase Test.[2]—*Principle.*—The amylase content of the urine is estimated. In nephritis the excretion of amylase is decreased.

[1] MacLean and deWesselow: *Brit. Jour. Exp. Path.*, 1, 1, 1920. See also McCaskey: *Med. Rec.*, 85, 507, 1914; Addis and Watenabe: *Jour. Biol. Chem.*, 28, 251, 1916, and Weiss: *Jour. Am. Med. Assn.*, 76, 298, 1921.

[2] MacLean: Modern Methods in the Diagnosis and Treatment of Renal Disease, London, 1921.

Procedure.—To a series of six test-tubes add amounts of the 24 hour urine[1] and of 0.9 per cent sodium chloride as indicated in the following table. Then add 2 c.c. of a 0.1 per cent solution of soluble starch to each tube. Shake quickly and put into an incubator or water bath at 37°C. for exactly 30 minutes. Remove tubes and fill to within an inch of the top with cold water. This slows up the digestion. Next add one or two drops of approximately N/10 iodine solution to each tube and mix. Note the number of the last tube to show no color or practically none with the iodine. From the tables obtain the unit value corresponding to this tube.

No.	Urine c.c.	Normal saline, c.c.	Unit value corresponding to each tube
1	1.0	...	2.0
2	0.6	0.4	3.3
3	0.4	0.6	5.0
4	0.3	0.7	6.6
5	0.2	0.8	10.0
6	0.1	0.9	20.0

Interpretation.—Normal urines usually show an activity of from 6 to 20 units. Values below 6 are suspicious. Values under 1 are found in advanced renal disease. This test should be used in conjunction with other tests as it sometimes gives erratic results.

Other tests for kidney function have been suggested by Kingsbury,[2] Volhard and Fahr[3] and Austin, Stillman and Van Slyke.[4]

[1] Dodds: *Brit. J. Exp. Path.* 3, 133, 1922 suggests prior dilution of the urine with 4 volumes of buffer solution made by mixing 15 c.c. of a solution containing 11.876 gm. $Na_2HPO_4 \cdot 2H_2O$ in a liter of water with 85 c.c. of a solution containing 9.078 gm. KH_2PO. in a liter, the mixture having a pH of 6.1.

[2] Kingsbury: *Arch. Int. Med.*, 32, 175, 1923.

[3] Volhard and Fahr: *Die Brightsche Nierenkrankheit*, Berlin, 1914.

[4] Austin, Stillman and Van Slyke: *Jour. Biol. Chem.*, 46, 91, 1921.

CHAPTER XXIX

URINE: ORGANIZED AND UNORGANIZED SEDIMENTS

The data obtained from carefully conducted microscopical examinations of the sediment of certain pathological urines are of very great importance diagnostically. Too little emphasis is sometimes placed upon the value of such findings.

The sedimentary constituents may be divided into two classes *i.e.*, *organized* and *unorganized*. The sediment is ordinarily collected for examination by means of the centrifuge (Fig. 201).[1] An older method, and one still in vogue in some quarters, is the so-called *gravity* method. This simply consists in placing the urine in a conical glass and allowing the sediment to settle. The collection of the sediment by means of the centrifuge, however, is much preferable, since the process of sedimentation may be accomplished by the use of this instrument in a few minutes, and far more perfectly, whereas when the other method is used it is frequently necessary to allow the urine to remain in the conical glass 12–24 hours

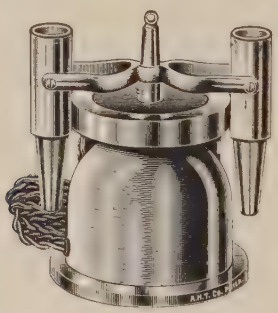


Fig. 201.—The Electric Centrifuge.

Fig. 202.—Conical Centrifuge Tube.

[1] The *supercentrifuge* (Sharples) is used for separating very fine suspensions such as bacteria in culture media or fat emulsions. It is constructed on the principle of a cream separator. For description of an *ultracentrifuge* used in determining molecular weights see Svedberg and Rinde, *Jour. Am. Chem. Soc.*, 46, 2677, 1924.

before sufficient sediment can be secured for the microscopical examination.

(a) Unorganized Sediments

Ammonium magnesium phosphate ("triple phosphate").
Calcium oxalate.
Calcium carbonate.
Calcium phosphate.
Calcium sulphate.
Uric acid.
Urates.
Cystine.
Cholesterol.
Hippuric acid.
Leucine and tyrosine.
Hematoidin and bilirubin.
Magnesium phosphate.
Indigo.
Xanthine.
Melanin.

Ammonium Magnesium Phosphate ("Triple Phosphate").—Crystals of "triple phosphate" are a characteristic constituent of the sediment when alkaline fermentation of the urine has taken place either *before* or after being voided. They may even be detected in amphoteric or *slightly* acid urine provided the ammonium salts are present in large enough quantity. This substance may occur in the sediment in two forms, *i.e.*, prisms and the feathery type. The prismatic form of crystals (Fig. 195, page 633) is the one most commonly observed in the sediment; the feathery form (Fig. 195, page 633) predominates when the urine is made ammoniacal with ammonia.

The sediment of the urine in such disorders as are accompanied by a retention of urine in the lower urinary tract contains "triple phosphate" crystals as a characteristic constituent. The crystals are frequently abundant in the sediment during paraplegia, chronic cystitis, enlarged prostate, and chronic pyelitis.

Calcium Oxalate.—Calcium oxalate is found in the urine in the form of at least two distinct types of crystals, *i.e.*, the *dumb-bell* type and the *octahedral type* (Fig. 203, page 682). Either form may occur in the sediment of neutral, alkaline, or acid urine, but both forms are found most frequently in urine having an acid reaction. Occasionally, in alkaline urine, the octahedral form is confounded with "triple phos-

phate" crystals. They may be differentiated from the phosphate crystals by the fact that they are insoluble in acetic acid.

The presence of calcium oxalate in the urine is not of itself a sign of any abnormality, since it is a constituent of normal urine. It is increased above the normal, however, in such pathological conditions as diabetes

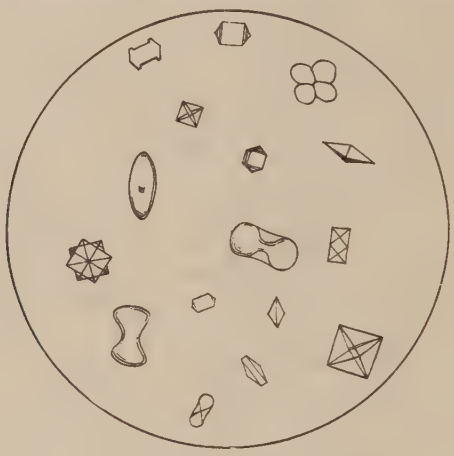


FIG. 203.—CALCIUM OXALATE. (*Ogden.*)

mellitus, in organic diseases of the liver, and in various other conditions which are accompanied by a derangement of digestion or of the oxidation mechanism, such as occurs in certain diseases of the heart and lungs.

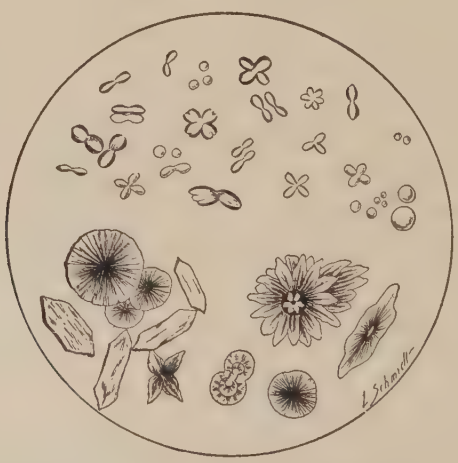


FIG. 204.—CALCIUM CARBONATE.

Calcium Carbonate.—Calcium carbonate crystals form a typical constituent of the urine of herbivorous animals. They occur less frequently in human urine. The reaction of urine containing these crystals is nearly always alkaline, although they may occur in ampho-

teric or in *slightly* acid urine. It generally crystallizes in the form of granules, spherules, or dumb-bells (Fig. 204). The crystals of calcium carbonate may be differentiated from calcium oxalate by the fact that they dissolve in acetic acid with the evolution of carbon dioxide gas.

Calcium Phosphate (Stellar Phosphate).—Calcium phosphate may occur in the urine in three forms, *i.e.*, amorphous, granular, or crystalline. The crystals of calcium phosphate are ordinarily pointed, wedge-shaped formations which may occur as individual crystals or grouped together in more or less regularly formed rosettes (Fig. 170, page 542). Acid sodium urate crystals (Fig. 206, page 685) are often mistaken for crystals of calcium phosphate. We may differentiate between these two crystalline forms by the fact that acetic acid will readily dissolve the phosphate, whereas the urate is much less soluble and when finally brought into solution and recrystallized one is frequently enabled to identify uric acid crystals which have been formed from the acid urate solution. The clinical significance of the occurrence of calcium phosphate crystals in the urinary sediment is similar to that of "triple phosphate" (see page 633).

Calcium Sulphate.—Crystals of calcium sulphate are of quite rare occurrence in the sediment of urine. Their presence seems to be limited in general to urines which are of a decided acid reaction. Ordinarily it crystallizes in the form of long, thin, colorless needles or prisms (Fig. 194, page 630) which may be mistaken for calcium phosphate crystals. There need be no confusion in this respect, however, since the sulphate crystals are insoluble in acetic acid, which reagent readily dissolves the phosphate. As far as is known their occurrence as a constituent of urinary sediment is of very little clinical significance.

Uric Acid.—Uric acid forms a very common constituent of the sediment of urines which are acid in reaction. It occurs in more varied forms than any of the other crystalline sediments (Plate VI, opposite page 602, and Fig. 205), some of the more common varieties of crystals being rhombic prisms, wedges, dumb-bells, whetstones, prismatic rosettes, irregular or hexagonal plates, etc. Crystals of pure uric acid are always colorless (Fig. 188, page 604), but the form occurring in urinary sediments is impure and under the microscope appears pigmented, the depth of color varying from yellow to a dark reddish-brown according to the size and form of the crystal.

The presence of a considerable uric acid sediment does not, of necessity, indicate a pathological condition or a urine of increased uric acid content, since this substance very often occurs as a sediment in urines whose uric acid content is diminished from the normal merely as a re-

sult of changes in reaction, etc. Pathologically, uric acid sediments occur in gout, acute febrile conditions, chronic interstitial nephritis, etc. If the microscopical examination is not conclusive, uric acid may be differentiated from other crystalline urinary sediments from the fact that it is soluble in alkalis, alkali carbonates, boiling glycerol, concentrated sulphuric acid, and in certain organic bases such as ethylamine and piperidin. It also responds to the murexide test (see page 604), Schiff's reaction (see page 605) and to Folin's phosphotungstic acid reaction (see page 604).

Urates.—The urate sediment may consist of a mixture of the urates of ammonium, calcium, magnesium, potassium, and sodium. The

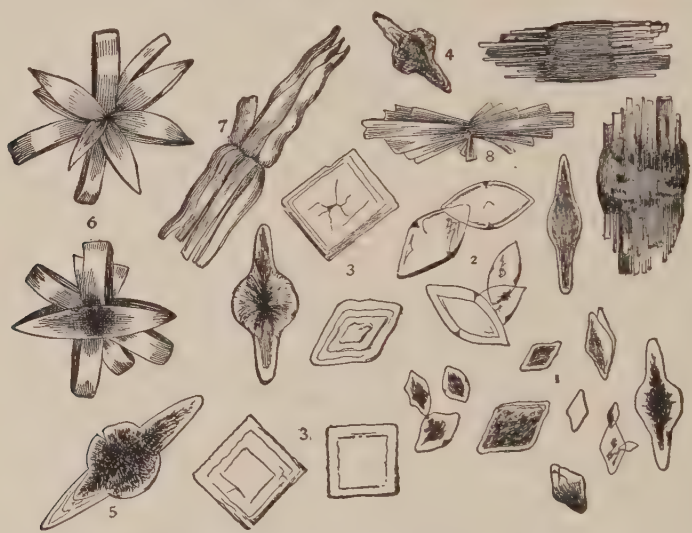


FIG. 205.—VARIOUS FORMS OF URIC ACID.

1, Rhombic plates; 2, whetstone forms; 3, 3, quadrate forms; 4, 5, prolonged into points; 6, 8, rosettes; 7, pointed bundles; 9, barrel forms precipitated by adding hydrochloric acid to urine.

ammonium urate may occur in neutral, alkaline, or acid urine, whereas the other forms of urates are confined to the sediments of acid urines. Sodium urate occurs in sediments more abundantly than the other urates. There are two sodium urates, the *mono* and the *di*, which may be expressed thus $\begin{matrix}Na^+\\H^+\end{matrix}\!\!>\!C_5H_2N_4O_3^=$ and $\begin{matrix}Na^+\\Na^+\end{matrix}\!\!>\!C_5H_2N_4O_3^=$. Both salts dissociate with the production of an alkaline reaction, the alkalinity being stronger in the case of the di-sodium urate. The so-called *quadriurate* or *hemiurate* have no existence as chemical units.[1] The urates of calcium, magnesium, and potassium are amorphous in character, whereas the urate of ammonium is crystalline. Sodium urate may be either amorphous or crystalline. When crystalline it

[1] Taylor: *Jour. Biol. Chem.*, 1, 177, 1905.

PLATE VII.

Ammonium Urates, Showing Spherules and Thorn-apple-shaped Crystals.
(From *Ogden*, after *Peyer*.)

forms groups of fan-shaped clusters or colorless, prismatic needles (Fig. 206). Ammonium urate is ordinarily present in the sediment in the burr-like form of the "thorn-apple" crystal, *i.e.*, yellow or reddish-brown spheres, covered with sharp spicules or prisms (Plate VII, opposite). The urates are all soluble in hydrochloric acid or acetic acid and their acid solutions yield crystals of uric acid upon standing. They also respond to the murexide test. The clinical significance of

FIG. 206.—ACID SODIUM URATE.

urate sediments is very similar to that of uric acid. A considerable sediment of amorphous urates does not necessarily indicate a high uric acid content, but ordinarily signifies a concentrated urine having a very strong acidity.

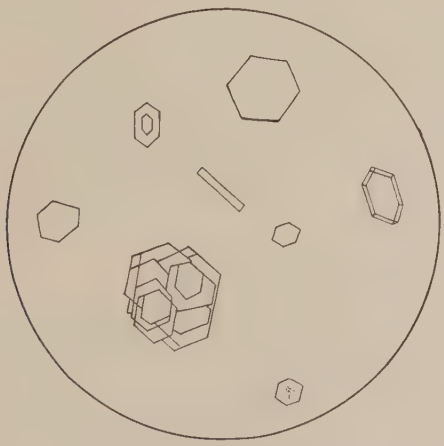


FIG. 207.—CYSTINE. (*Ogden.*)

Cystine.—Cystine is one of the rarer of the crystalline urinary sediments. It has been claimed that it occurs more often in the urine of

men than of women. Cystine crystallizes in the form of thin, colorless, hexagonal plates (Fig. 43, page 143, and Fig. 207) which are insoluble in water, alcohol, and acetic acid, and soluble in mineral acids, alkalis, and especially in ammonia. Cystine may be identified by burning it upon platinum foil, under which condition it does not melt but yields a bluish-green flame. For preparation of Cystine see page 143.

Cholesterol.—Cholesterol crystals have been but rarely detected in urinary sediments. When present they probably arise from a pathological condition of some portion of the urinary tract. Crystals of cholesterol have been found in the sediment in cystitis, pyelitis, chyluria, and nephritis. Ordinarily it crystallizes in large regular and irregular colorless, transparent plates, some of which possess notched corners (Fig. 81, page 281). Frequently, instead of occurring in the sediment, it is found in the form of a film on the surface of the urine.

Hippuric Acid.—This is one of the rare sediments of human urine. It deposits under conditions similar to those which govern the formation of uric acid sediments. The crystals, which are colorless needles or prisms (Fig. 191, page 613) when pure, are invariably pigmented in a manner similar to the uric acid crystals when observed in urinary sediment and because of this fact are frequently confounded with the rarer forms of uric acid. Hippuric acid may be differentiated from uric acid from the fact that it does not respond to the murexide test and is much more soluble in water and in ether. The detection of crystals of hippuric acid in the urine has very little clinical significance, since its presence in the sediment depends in most instances very greatly upon the nature of the diet. It is particularly prone to occur in the sediment after the ingestion of certain fruits as well as after the ingestion of benzoic acid (see Chapter XXXII).

FIG. 208.—CRYSTALS OF IMPURE LEUCINE. (*Ogden.*)

Leucine and Tyrosine.—Leucine and tyrosine have frequently been detected in the urine, either in solution or as a sediment. Neither of them occurs in the urine ordinarily except in association with the others, *i.e.*, whenever leucine is detected it is more than probable that tyrosine accompanies it. They have been found pathologically in the urine in acute yellow atrophy of the liver, in acute phosphorus poisoning, in cirrhosis of the liver, in severe cases of typhoid fever and small-pox, and in leukemia. In urinary sediments leucine ordi-

narily crystallizes in characteristic spherical masses which show both radial and concentric striations and are highly refractive (Fig. 208). Some investigators claim that these crystals which are ordinarily called leucine are, in reality, generally urates. For the crystalline form of pure leucine obtained as a decomposition product of protein see Fig. 38, page 134. Tyrosine crystallizes in urinary sediments in the well-known sheaf or tuft formation (Fig. 46, page 148). For other tests on leucine and tyrosine see pages 134 and 150.

Hematoidin and Bilirubin.—There are divergent opinions regarding the occurrence of these bodies in urinary sediment. Each of them crystallizes in the form of tufts of small needles or in the form of small plates which are ordinarily yellowish-red in color (Fig. 80, page 276). Because of the fact that the crystalline form of the two substances is identical many investigators claim them to be one and the same body. Other investigators claim, that while the crystalline form is the same in each case, there are certain chemical differences which may be brought out very strikingly by properly testing. For instance, it has been claimed that hematoidin may be differentiated from bilirubin through the fact that it gives a momentary color reaction (blue) when nitric acid is brought into contact with it, and, further, that it is not dissolved on treatment with ether or potassium hydroxide. Pathologically, typical crystals of hematoidin or bilirubin have been found in the urinary sediment in jaundice, acute yellow atrophy of the liver, carcinoma of the liver, cirrhosis of the liver, and in phosphorus poisoning, typhoid fever, and scarlatina.

Magnesium Phosphate.—Magnesium phosphate crystals occur rather infrequently in the sediment of urine which is neutral, alkaline, or *feebly* acid in reaction. It ordinarily crystallizes in elongated, highly refractive, rhombic plates which are soluble in acetic acid.

Indigo.—Indigo crystals are frequently found in urine which has undergone alkaline fermentation. They result from the breaking down of indoxyl-sulphates or indoxyl-glycuronates. Ordinarily indigo deposits as dark blue stellate needles or occurs as amorphous particles or broken fragments. These crystalline or amorphous forms may occur in the sediment or may form a blue film on the surface of the urine. Indigo crystals generally occur in urine which is alkaline in reaction, but they have been detected in acid urine.

Xanthine.—Xanthine is a constituent of normal urine but is found in the sediment in crystalline form very infrequently, and then only in pathological urine. When present in the sediment xanthine generally occurs in the form of whetstone-shaped crystals somewhat similar in form to the whetstone variety of uric acid crystal. They may be dif-

ferentiated from uric acid by the great ease with which they may be brought into solution in dilute ammonia and on applying heat. Xanthine may also form urinary calculi. The clinical significance of xanthine in urinary sediment is not well understood.

Melanin.—Melanin is an extremely rare constituent of urinary sediments. Ordinarily in melanuria the melanin remains in solution; if it separates it is generally held in suspension as fine amorphous granules.

(b) Organized Sediments

Epithelial cells.
Pus cells.
Casts.
- Hyaline.
- Granular.
- Epithelial.
- Blood.
- Fatty.
- Waxy.
- Pus.

Cylindroids.
Erythrocytes.
Spermatozoa.
Urethral filaments.
Tissue débris.
Animal parasites.
Micro-organisms.
Fibrin.
Foreign substances due to contamination.

Epithelial Cells.—The detection of a certain number of these cells in urinary sediment is not, of itself, a pathological sign, since they occur in normal urine. However, in certain pathological conditions they are greatly *increased* in number, and since different areas of the urinary tract are lined with different forms of epithelial cells, it becomes necessary, when examining urinary sediments, to note not only the relative number of such cells, but at the same time to carefully observe the shape of the various individuals in order to determine, as far as possible, from what portion of the tract they have been derived. Since the different layers of the epithelial lining are composed of cells different in form from those of the associated layers, it is evident that a careful microscopical examination of these cells may tell us the particular layer which is being desquamated. It is frequently a most difficult undertaking, however, to make a clear differentiation between the various forms of epithelial cells present in the sediment. If skillfully

done, such a microscopical differentiation may prove to be of very great diagnostic aid.

The principal forms of epithelial cells met with in urinary sediments are shown in Fig. 209, below.

Pus Cells.—Pus corpuscles or leucocytes are present in extremely small numbers in normal urine. Any considerable increase in the number, however, ordinarily denotes a pathological condition, generally an acute or chronic inflammatory condition of some portion of the urinary tract. The sudden appearance of a large amount of pus in a sediment denotes the opening of an abscess into the urinary tract. Other form elements, such as epithelial cells, casts, etc., ordinarily

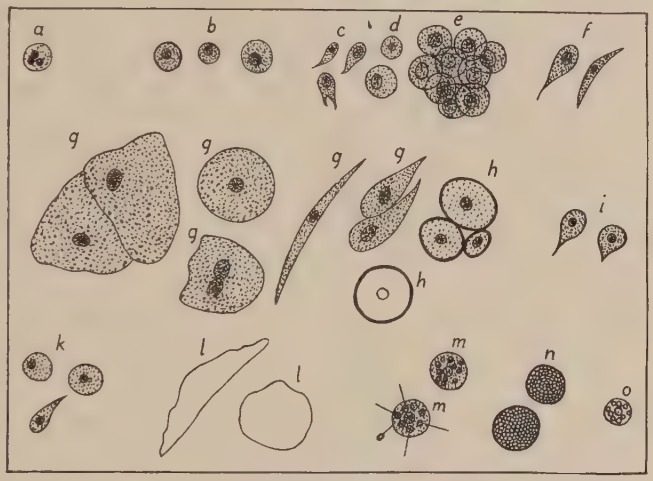


FIG. 209.—EPITHELIUM FROM DIFFERENT AREAS OF THE URINARY TRACT.

a, Leucocyte (for comparison); *b*, renal cells; *c*, superficial pelvic cells; *d*, deep pelvic cells; *e*, cells from calices; *f*, cells from ureter; *g*, *g*, *g*, *g*, *g*, squamous epithelium from the bladder; *h*, *h*, neck-of-bladder cells; *i*, epithelium from prostatic urethra; *k*, urethral cells; *l*, *l*, scaly epithelium; *m*, *m'*, cells from seminal passages; *n*, compound granule cells; *o*, fatty renal cell. (*Ogden.*)

accompany pus corpuscles in urinary sediment and a careful examination of these associated elements is necessary in order to form a correct diagnosis as to the origin of the pus. Protein is always present in urine which contains pus.

The appearance which pus corpuscles exhibit under the microscope depends greatly upon the reaction of the urine containing them. In acid urine they generally present the appearance of round, colorless cells composed of refractive, granular protoplasm, and may frequently exhibit amœboid movements, especially if the slide containing them be warmed slightly. They are nucleated (one or more nuclei), the nuclei being clearly visible only upon treating the cells with water, acetic acid, or some other suitable reagent. In urine which has a decided alkaline

reaction, on the other hand, the pus corpuscles are often greatly degenerated. They may be seen as swollen, transparent cells, which exhibit no granular structure and as the process of degeneration continues the cell outline ceases to be visible, the nuclei fade, and finally only a mass of débris containing isolated nuclei and an occasional cell remains.

It is frequently rather difficult to make a differentiation between pus corpuscles and certain types of epithelial cells which are similar in form. Such confusion may be avoided by the addition of iodine solution (I in KI), a reagent which stains the pus corpuscles a deep mahogany-brown and transmits to the epithelial cells a light yellow tint. The test pro-

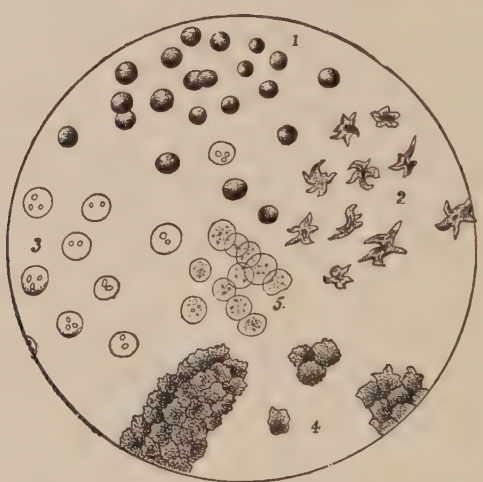


FIG. 210.—PUS CORPUSCLES. (After *Ultzmann.*)
1, Normal; 2, showing amœboid movements; 3, nuclei rendered distinct by acetic acid; 4, as observed in chronic pyelitis; 5, swollen by ammonium carbonate.

posed by Vitali often gives very satisfactory results. This simply consists in acidifying the urine (if alkaline) with acetic acid, then filtering, and treating the sediment on the filter paper with freshly prepared tincture of guaiac. The presence of pus in the sediment is indicated if a *blue* color is observed. Large numbers of pus corpuscles are present in the urinary sediment in gonorrhœa, leucorrhœa, chronic pyelitis, and in abscess of the kidney. In addition to the usual constituents found in leucocytes Mandel and Levene[1] claim that pus cells contain *glucothionic acid.* See Pus tests, page 653.

Casts.—These are cylindrical formations, which originate in the uriniferous tubules and are forced out by the pressure of the urine. They vary greatly in size, but in nearly every instance they possess parallel sides and rounded ends. The finding of casts in the urine is

[1] Mandel and Levene: *Biochemische Zeitschrift,* 4, 78, 1907.

very important because of the fact that they *generally indicate* some kidney disorder; if albumin accompanies the casts the indication is much accentuated. Casts have been classified according to their microscopical characteristics as follows: (*a*) hyaline, (*b*) granular, (*c*) epithelial, (*d*) blood, (*e*) fatty, (*f*) waxy, (*g*) pus.

(*a*) *Hyaline Casts.*—These are composed of a basic material which is transparent, homogeneous, and very light in color (Fig. 211). In fact, chiefly because of these physical properties, they are the most difficult form of renal casts to detect under the microscope. Frequently such casts are impregnated with deposits of various forms,

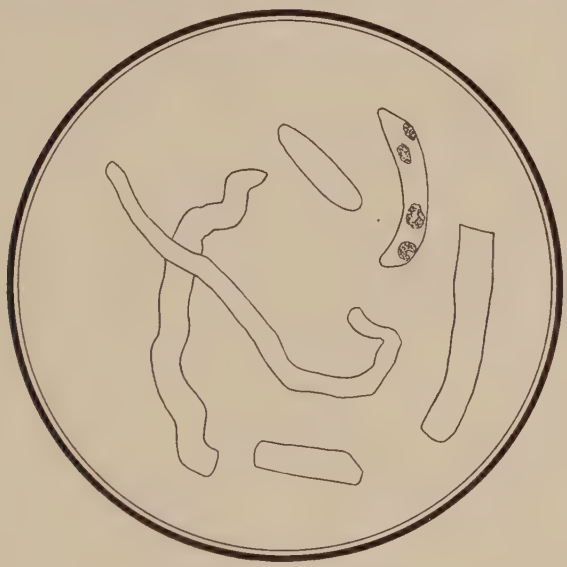


FIG. 211.—HYALINE CASTS.
One cast is impregnated with four renal cells.

such as erythrocytes, epithelial cells, fat globules, etc., thus rendering the form of the cast more plainly visible. Staining is often resorted to in order to render the shape and character of the cast more easily determined. Ordinary iodine solution (I in KI) may be used in this connection; many of the aniline dyes are also in common use for this purpose, *e.g.*, gentian-violet, Bismarck-brown, methylene-blue, fuchsin, and eosin. Generally, but not always, albumin is present in urine containing hyaline casts. Hyaline casts are common to all kidney disorders, but occur particularly in the earliest and recovering stages of parenchymatous nephritis and interstitial nephritis.

(*b*) *Granular Casts.*—The common hyaline material is ordinarily the basic substance of this form of cast. The granular material generally consists of albumin, epithelial cells, fat, or disintegrated erythrocytes or

leucocytes, the character of the cast varying according to the nature and size of the granules (Fig. 212, and Fig. 213 below). Thus we have casts of this general type classified as *finely granular* and *coarsely granular* casts. Granular casts, and in particular the finely granular types, occur in the sediment in practically every kidney disorder but are probably especially characteristic of the sediment in inflammatory disorders.

(*c*) *Epithelial Casts.*—These are casts bearing upon their surface epithelial cells from the lining of the uriniferous tubules (Fig. 214, page 693). The basic material of this form of cast may be hyaline or

FIG. 212.—GRANULAR CASTS. (After *Peyer.*)

granular in nature. Epithelial casts are particularly abundant in the urinary sediment in *acute* nephritis.

(*d*) *Blood Casts.*—Casts of this type may consist of erythrocytes borne upon a hyaline or a fibrinous basis (Fig. 215, page 693). The occurrence of such casts in the urinary sediment denotes renal hemorrhage and they are considered to be especially characteristic of acute diffuse nephritis and acute congestion of the kidney.

(*e*) *Fatty Casts.*—Fatty casts may be formed by the deposition of fat globules or crystals of fatty acid upon the surface of a hyaline or granular cast (Fig. 216, page 694). In order to constitute a true fatty cast the deposited material must cover the greater part of the surface area of the cast. The presence of fatty casts in urinary sediment in-

dicates fatty degeneration of the kidney; such casts are particularly characteristic of subacute and chronic inflammation of the kidney.

FIG. 213.—GRANULAR CASTS.
a, Finely granular; *b*, coarsely granular.

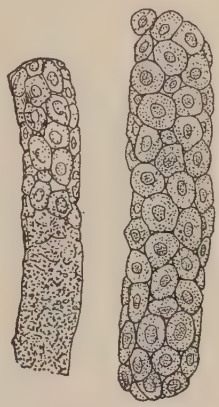


FIG. 214.—EPITHELIAL CASTS.

(*f*) *Waxy Casts.*—These casts possess a basic substance similar to that which enters into the foundation of the hyaline form of cast. In

FIG. 215.—BLOOD, PUS, HYALINE AND EPITHELIAL CASTS.
a, Blood casts; *b*, pus cast; *c*, hyaline cast impregnated with renal cells; *d*, epithelial casts.

common with the hyaline type they are colorless, refractive bodies, but differ from this form of cast in being, in general, of greater length and diameter and possessing sharper outlines and a light yellow color

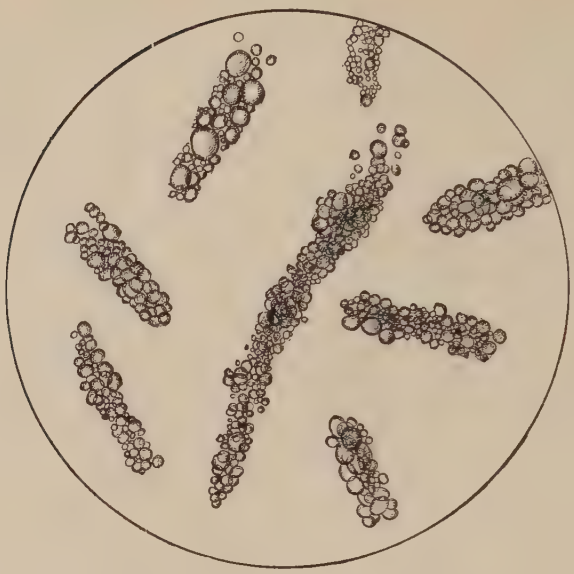


FIG. 216.—FATTY CASTS. (After *Peyer*.)

FIG. 217.—FATTY AND WAXY CASTS.
a, Fatty casts; *b*, waxy casts.

(Fig. 217, page 694). Such casts occur in several forms of nephritis, but do not appear to characterize any particular type of the disorder except *amyloid disease*, in which they are rather common.

(*g*) *Pus Casts.*—Casts whose surface is covered with pus cells or leucocytes are termed *pus casts* (Fig. 215, p. 693). They are frequently mistaken for epithelial casts. The differentiation between these two types is made very simple, however, by treating the cast with acetic acid which causes the nuclei of the leucocytes to become plainly visible. The true pus cast is quite rare and indicates renal suppuration.

Cylindroids.—These formations may occur in normal or pathological urine and have no particular clinical significance. They are frequently

FIG. 218.—CYLINDROIDS. (After *Peyer.*)

mistaken for true casts, especially the hyaline type, but they are ordinarily *flat* in structure with a rather smaller diameter than casts, may possess forked or branching ends, and are not composed of homogeneous material as are the hyaline casts. Such "false casts" may become coated with urates, in which event they appear granular in structure. The basic substance of cylindroids is often the nucleoprotein of the urine (Fig. 218, above).

Erythrocytes.—These form elements are present in the urinary sediment in various diseases. They appear as the normal biconcave, yellow erythrocyte (Plate V, opposite page 335) or may exhibit certain modifications in form, such as the crenated type (Fig. 219) which is often seen in concentrated urine. Under different condi-

tions they may become swollen sufficiently to entirely erase the biconcave appearance and may even occur in the form of colorless spheres having a smaller diameter than the original disc-shaped corpuscles. Erythrocytes are found in urinary sediment in hemorrhage of the kidney or of the urinary tract, in traumatic hemorrhage, hemorrhage from congestion, and in hemorrhagic diathesis.

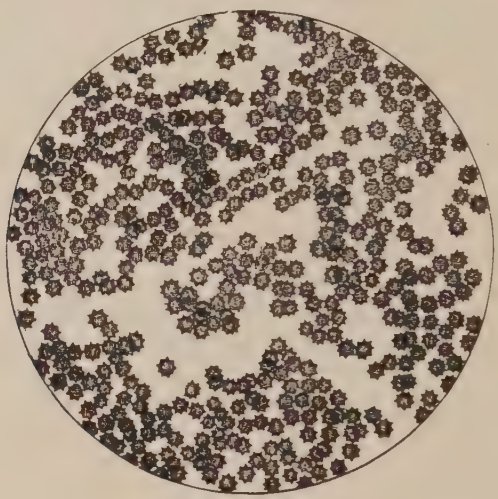


FIG. 219.—CRENATED ERYTHROCYTES.

Spermatozoa.—Spermatozoa may be detected in the urinary sediment in diseases of the genital organs, as well as after coitus, nocturnal emissions, epileptic, and other convulsive attacks, and sometimes in severe febrile disorders, especially in typhoid fever. In form they consist of an oval body, to which is attached a long, delicate tail (Fig. 220, page above). Upon examination they may show motility or may be motionless.

FIG. 220.—HUMAN SPERMATOZOA.

Urethral Filaments.—These are peculiar thread-like bodies which are sometimes found in urinary sediment. They may occasionally be detected in normal urine and pathologically are found in the sediment in acute and chronic gonorrhœa and in urethrorrhœa. The ground-substance of these urethral filaments is, in part at least, similar to that of the cylindroids (see page 695). The urine first voided in the morning is best adapted for the examination for filaments. These filaments may ordinarily be removed by a pipette since they are generally macroscopic.

Tissue Débris.—Masses of cells or fragments of tissue are frequently found in the urinary sediment. They may be found in the sediment in tubercular affections of the kidney and urinary tract or in tumors of these organs. Ordinarily it is necessary to make a histological examination of such tissue fragments before coming to a final decision as to their origin.

Animal Parasites.—The cysts, hooklets, and membrane shreds of *echinococci* are sometimes found in the urinary sediments. Other animal organisms which are more rarely met with in the urine are embryos of the *Filaria sanguinis* and eggs of the *Distoma hematobium* and *Ascarides*. Animal parasites in general occur most frequently in the urine in tropical countries.

Micro-organisms.—Bacteria as well as yeasts and moulds are frequently detected in the urine. Both the pathogenic and non-pathogenic forms of bacteria may occur. The non-pathogenic forms most frequently observed are *micrococcus ureæ*, *bacillus ureæ*, and *staphylococcus ureæ liquefaciens*. Of the pathogenic forms many have been observed, *e.g.*, *Bacterium Coli*, *typhoid bacillus*, *tubercle bacillus*, *gonococcus*, *bacillus pyocyaneus*, and *proteus vulgaris*. Yeast and moulds are most frequently met with in diabetic urine.

Fibrin.—Following hematuria, fibrin clots are occasionally observed in the urinary sediment. They are generally of a semi-gelatinous consistency and of a very light color, and when examined under the microscope they are seen to be composed of bundles of highly refractive fibers which run parallel.

Foreign Substances Due to Contamination.—Such foreign substances as fibers of silk, linen, or wool; starch granules, hair, fat, and sputum, as well as muscle fibers, vegetable cells, and food particles, are often found in the urine. Care should be taken that these foreign substances are not mistaken for any of the true sedimentary constituents already mentioned.

CHAPTER XXX

URINE: CALCULI

URINARY *calculi*, also called *concretions*, or *concrements* are solid masses of urinary sediment formed in some part of the urinary tract. They vary in shape and size according to their location, the smaller calculi, termed *sand* or *gravel*, in general arising from the kidney or the pelvic portion of the kidney, whereas the large calculi are ordinarily formed in the bladder. There are two general classes of calculi as regards composition, *i.e.*, *simple* and *compound*. The simple form is made up of but a single constituent, whereas the compound type contains two or more individual constituents. The structural plan of most calculi consists of an arrangement of concentric rings about a central nucleus, the number of rings frequently being dependent upon the number of individual constituents which enter into the structure of the calculus. However, layers quite different in macroscopical appearance may be almost identical in composition. In case two or more calculi unite to form a single calculus the resultant body will obviously contain as many nuclei as there were individual calculi concerned in its construction. Under certain conditions the growth of a calculus will be principally in only one direction, thus preventing the nucleus from maintaining a central location. The qualitative composition of urinary calculi is dependent, in great part, upon the reaction of the urine, *e.g.*, if the reaction of the urine is acid the calculi present will be composed, in great part at least, of substances that are capable of depositing in acid urine, *e.g.*, uric acid, urates and calcium oxalate.

According to Ultzmann, out of 545 cases of urinary calculus, uric acid and urates formed the nucleus in about 81 per cent of the cases; earthy phosphates in about 9 per cent; calcium oxalate in about 6 per cent; cystine in something over 1 per cent, while in about 3 per cent of the cases some foreign body comprised the nucleus.

More recent analyses[1] of twenty-four calculi showed the nucleus in 75 per cent of them to be calcium oxalate (60 per cent) and in 25 per cent to be phosphate (56 per cent). All of the calculi contained some uric acid and urates, but only three gave more than 10 per cent.

[1] Kahn and Rosenbloom: *Jour. Am. Med. Ass'n*, 59, 2252, 1913.

In the chemical examination of urinary calculi the most valuable data are obtained by subjecting each of the concentric layers of the calculus to a separate analysis. Material for examination may be conveniently obtained by sawing the calculus carefully through the nucleus, then separating the various layers, or by scraping off from each layer (without separating the layers) enough powder to conduct the examination as outlined in the scheme (see page 700).

Varieties of Calculus

Uric Acid and Urate Calculi.—Uric acid and urates constitute the nuclei of a large proportion of urinary concretions. Such stones are always colored, the tint varying from a pale yellow to a brownish-red. The surface of such calculi is generally smooth but it may be rough and uneven.

Phosphatic Calculi.—Ordinarily these concretions consist principally of "triple phosphate" and other phosphates of the alkaline earths, with very frequent admixtures of urates and oxalates. The surface of such calculi is generally rough but may occasionally be rather smooth. The calculi are somewhat variable in color, exhibiting gray, white, or yellow tints under different conditions. When composed of earthy phosphates the calculi are characterized by their friability.

Calcium Oxalate Calculi.—This is the hardest form of calculus to deal with, and is rather difficult to crush. They ordinarily occur in two general forms, *i.e.*, the small, smooth concretion which is characterized as the *hemp-seed calculus*, and the medium-sized or large stone possessing an extremely uneven surface, which is generally classed as a *mulberry calculus*. This roughened surface of the latter form of calculus is due, in many instances, to protruding calcium oxalate crystals of the octahedral type.

Calcium Carbonate Calculi.—Calcium carbonate concretions are quite common in herbivorous animals, but of exceedingly rare occurrence in man. They are generally small, white, or grayish calculi, spherical in form and possess a hard, smooth surface.

Cystine Calculi.—The cystine calculus is a rare variety of calculus. Ordinarily they occur as small, smooth, oval, or cylindrical concretions which are white or yellow in color and of a rather soft consistency.

Xanthine Calculi.—This form of calculus is somewhat more rare than the cystine type. The color may vary from white to brownish-yellow. Very often uric acid and urates are associated with xanthine in this type of calculus. Upon rubbing a xanthine calculus it has the property of assuming a wax-like appearance.

On Heating the Powder on Platinum Foil, It							
Does burn			Does not burn				
With flame		Without flame	The powder when treated with HCl				
			Does not effervesce	The powder gently heated, then treated with HCl		The powder when moistened with a little KOH	
						No ammonia or, at least, only traces of ammonia. Powder dissolves in acetic acid or HCl. This solution is precipitated by ammonia (amorphous)	Abundant ammonia. The powder dissolves in acetic acid or HCl. This solution gives a crystalline precipitate with ammonia
					Effervesces		
			Effervesces				
				Calcium carbonate.			
				Calcium oxalate.			
						Bone-earth (magnesium and calcium phosphate).	
							"Triple phosphate" (mixed with unknown amount of earthy phosphate).

Urostealith Calculi.—This form of calculus is extremely rare. Such concretions are composed principally of fat and fatty acid. When moist they are soft and elastic, but when dried they become brittle. Urostealiths are generally light in color.

Fibrin Calculi.—Fibrin calculi are produced in the process of blood coagulation within the urinary tract. They frequently occur as nuclei of other forms of calculus. They are rarely found.

Cholesterol Calculi.—An extremely rare form of calculus somewhat resembling the cystine type.

Indigo Calculi.—Indigo calculi are extremely rare, only two cases having been reported.

The scheme, proposed by Heller and given on page 700, will be found of much assistance in the chemical examination of urinary calculi.

CHAPTER XXXI

URINE: QUANTITATIVE ANALYSIS

In analyzing a normal or pathological urine quantitatively for any of its constituents it is particularly necessary that the complete and exact 24-hour sample be obtained. For directions with regard to the collection and preservation of urine for analysis see Chapter XXVI on General Characteristics of Normal and Pathological Urine. Methods for the determination of the specific gravity of the urine are also there described. Before any urine is taken for analysis its total volume should be measured, using a large graduated cylinder, and this volume is thereafter taken as a basis for the calculations of the daily output of the individual constituents determined. If the urine be pathological it is of course necessary to precede its quantitative analysis by qualitative tests for the pathological constituents.

PREPARATION OF STANDARD ACID AND ALKALI SOLUTIONS

Principle.--Many of the quantitative methods used in physiological chemistry are volumetric or titration procedures. For these methods solutions of accurately known strength called *standard* solutions are needed. Their strength is usually expressed in terms of normality. A *normal* solution is one which contains in 1000 c.c. one gram of replaceable hydrogen or its equivalent. Thus to make 1000 c.c. of a normal solution of hydrochloric acid (HCl), we would need 36.5 grams of this acid containing one gram of replaceable hydrogen. This we derive from the fact that the atomic weight of Cl is 35.5 and of H is 1, so that the molecular weight of HCl is 36.5, and each 36.5 grams of this acid contain 1 gram of replaceable hydrogen.[1] Sulphuric acid (H_2SO_4) has a molecular weight of 98 or (2 + 32 + 64), but 98 grams of sulphuric acid contain 2 grams of replaceable hydrogen. Therefore, to prepare a normal solution of this acid, we must use one half of 98 or 49 grams of sulphuric acid (containing one gram of hydrogen) for 1000 c.c. of normal solution. Oxalic acid ($H_2C_2O_4 + 2H_2O$) has a molecular weight of 126 or (2 + 24 + 64 + 36). It also is a dibasic acid so we must use only one-half of 126 or 63 grams of oxalic acid in making a liter of normal solution.

[1] See table of atomic weights, Appendix.

A normal alkali solution is exactly equivalent to a normal acid solution, *i.e.*, a liter of the alkali will neutralize a liter of the acid. According to the reaction of neutralization, therefore, the 36.5 grams of HCl in a liter of this normal acid will require 40.0 grams of sodium hydroxide to neutralize it, and a liter of normal sodium hydroxide must contain 40.0 grams of the alkali.

$$\underset{(36.5)}{HCl} + \underset{(40.0)}{NaOH} \rightarrow \underset{(58.5)}{NaCl} + \underset{(18)}{H_2O}$$

Having prepared solutions of acid and alkali of definitely known strength, it is then possible to determine the strength of any unknown acid or alkali by finding out how much of these standard solutions is required to neutralize a definite volume of the unknown solution.

In order to tell when the unknown solution has been exactly neutralized, we use a small amount of one of a class of substances called *indicators*. An indicator changes color at, or near, the neutral point, and this color change indicates the end point of the titration.

When strong acids (as HCl) are being titrated with strong alkalies (as NaOH), almost any one of the common indicators is satisfactory. If weak acids (acetic acid) or weak bases (as ammonia) are being titrated, it is necessary to be very careful in the choice of an indicator as all indicators are not sufficiently sensitive to those.[1]

Preparation of N/10 Oxalic Acid Solution.—Weigh accurately a watch glass or a piece of glazed paper. Then add to the weights on the balance pan 3.1512 gm. With a spatula transfer to the watch glass enough pure oxalic acid in the form of clear crystals to counterbalance exactly the weights in the opposite pan. Transfer completely to a 250 c.c. beaker with the aid of a camel's hair brush. Add about 150 c.c. of distilled water and stir with a glass rod until dissolved, warming gently if necessary. Transfer every particle of this solution to a clean 500 c.c. volumetric flask, rinsing rod and beaker several times with distilled water. Hold under the tap until cooled to room temperature. Then add distilled water until the bottom of the meniscus is level with the mark on the neck of the flask (the lower mark if there are two). Insert a stopper and mix thoroughly by inverting the flask again and again. Transfer to a clean dry bottle. Label. This solution will not keep indefinitely and is to be used only in the standardization of N/10 alkali.

Preparation of N/10 Sodium Hydroxide Solution.—(a) Preparation of concentrated carbonate-free sodium hydroxide solution.—Shake up about 110 gm. best quality NaOH with 100 c.c. of distilled water in a 300 c.c. Erlenmeyer flask (Pyrex) to make a saturated solution. Stopper and allow to stand for a couple of days or until the sodium carbonate settles to the bottom leaving a clear solution of NaOH practically free from carbonate.

(b) Preparation of a Standard Sodium Hydroxide Solution.—Measure out 6.3 c.c. of the saturated NaOH solution from a burette into a liter flask. Add

[1] For a further consideration of indicators see Chapter I.

750 c.c. of distilled water and mix thoroughly. Clean a burette by allowing it to stand filled with cleaning mixture (potassium dichromate and sulphuric acid) for a few minutes or longer if necessary. Empty, rinse several times with tap water, finally with distilled water, and allow to drain. If necessary to use the burette before it is perfectly dry, introduce a few c.c. of the NaOH solution, and invert a couple of times to rinse the burette, discarding this NaOH. Then fill the burette with the alkali solution, making sure that the tip contains no air bubbles, and run out solution until the bottom of the meniscus is exactly at 0.

Into a clean Erlenmeyer flask (150–250 c.c.) now introduce 25 c.c. of N/10 oxalic acid solution measured from an accurate, clean pipette, previously rinsed by means of a little of the acid solution drawn up into it. Allow the pipette to drain about 15 seconds against the side of the flask. Add 2-3 drops of a 1 per cent alcoholic solution of phenolphthalein.

Now run in NaOH solution from the burette, rotating the flask. Ten c.c. can be added quite rapidly; then add more slowly, and finally drop by drop until the last drop changes the color of the solution permanently throughout to a definite pink. Take the burette reading. Repeat the titration until two exact duplicate readings are obtained.

Calculate the strength of the NaOH solution. Divide 25 (the number of c.c. of N/10 oxalic acid used) by the burette reading and obtain the strength of the NaOH in terms of N/10 solution. For example, if 15.6 c.c. were required: $25 \div 15.6 = 1.603 \times 0.1 = 0.1603$ N.

(c) Preparation of the N/10 NaOH Solution.—Calculate how much of the standard NaOH solution just prepared will be required to make a liter of N/10 solution. To do this divide 1000 c.c. by the strength of the NaOH in terms of N/10 solution. Thus in the example cited above: $1000 \div 1.603 = 623.8$ c.c. required. Measure out the exact amount of alkali required (using the burette, pipette, and volumetric flasks) into a 1000 c.c. flask. Dilute with distilled water exactly to the mark. Mix very thoroughly and transfer to a clean, dry bottle with a rubber (not glass) stopper. Check the strength of the solution by again titrating 25 c.c. portions of oxalic acid solution.[1]

Preparation of N/10 Hydrochloric Acid.—Concentrated hydrochloric acid is about 10 N or 36.5 per cent HCl. Approximately N/10 HCl may, therefore, be prepared by diluting 10 c.c. of the concentrated acid to 1 liter in a volumetric flask. This must be standardized by titration with N/10 alkali, using preferably alizarin red as an indicator.

Or introduce into a liter flask 12 c.c. of concentrated HCl and 750 c.c. of distilled water. Mix well and titrate 10, 15, or 25 c.c. portions of the acid solution with N/10 NaOH, using alizarin as an indicator. Dividing the number of c.c. of acid used by the number of c.c. of N/10 NaOH required gives the strength of the HCl in terms of N/10 solution. Dividing 1000 by this quotient gives the number of c.c. of HCl solution to be measured into a volumetric flask and made up to 1000 c.c.

This diluted solution will be N/10 HCl. It should be mixed thoroughly and 25 c.c. portions of it checked by titration with the N/10 NaOH.[2]

[1] If a very high degree of accuracy is desired, the alkali may be checked against pure acid potassium phthalate.

[2] The acid solution may be standardized directly in the following manner: Introduce a platinum dish containing very pure sodium bicarbonate or the highest grade anhydrous sodium carbonate into a hot air oven previously heated to 200°C. Raise the temperature

Standard acid and alkali solutions are best kept in paraffin-lined bottles. The acid solution is the more permanent of the two. Alkali solutions must be protected from the carbonic acid of the air, the solution being best drawn over into the burette by means of a siphon tube leading from the top of the burette to the interior of the alkali bottle. The air inlet through the stopper of the bottle should be guarded by a tube containing soda lime.

Preparation of Standard Hydrochloric Acid from a Constant Boiling Solution. Method of Hulett and Bonner.[1] Procedure.—Make up by hydrometer about 200 c.c. of HCl of specific gravity about 1.10. Distill off about ¾ of the liquid and discard. Then collect about 25 c.c. of the constant boiling distillate. Weigh 18.017 gm. of distillate using a capillary pipette for final adjustment (or measure out 16.442 c.c. at 25°C.). Dilute to 1 liter. This is a 0.1 N solution of HCl. The figures given are for a barometric pressure of 760 mm. At 770 mm. use 18.039 gm.; at 750 mm. use 17.996 gm.; at 740 mm. use 17.975 gm. and at 730 mm. use 17.953 gm. The composition of the distillate should not vary more than 1 part in 10,000 from the figures given.

Acidity by Titration

Folin's Method.—*Principle.*—The urine is titrated with standard sodium hydroxide solution, using phenolphthalein as an indicator. Potassium oxalate is added to precipitate the calcium which would otherwise interfere with the end-point due to the precipitation of calcium phosphate on neutralization of the urine. The acidity of the urine as determined in this way is not a correct measure of the true acidity, which is dependent upon the concentration of hydrogen ions. The results obtained do, however, ordinarily show a certain parallelism with the hydrogen ion concentration and are of value for comparative purposes.

Procedure.—Place 25 c.c. of urine in a 200 c.c. Erlenmeyer flask and add 15–20 grams of finely pulverized potassium oxalate and 1–2 drops of a 1 per cent phenolphthalein solution to the fluid. Shake the mixture vigorously for 1–2 minutes and titrate it immediately with N/10 sodium hydroxide until a faint but unmistakable pink remains permanent on further shaking. Take the burette reading and calculate the acidity of the urine under examination.

Calculation.—If y represents the number of cubic centimeters of N/10 sodium hydroxide used and y′ represents the volume of urine excreted in 24 hours, the total acidity of the 24-hour urine specimen may be calculated by means of the following proportion:

$$25 : y :: y' : x$$ (acidity of 24-hour urine expressed in cubic centimeters of N/10 sodium hydroxide).

Each cubic centimeter of N/10 sodium hydroxide contains 0.004 gram of sodium hydroxide, and this is equivalent to 0.0063 gram of oxalic acid. There-

to 270°–280°, but not above 300°C. Heat for half an hour, allow to cool in a desiccator, but while still a little warm, transfer to a glass stoppered weighing bottle. Weigh out rapidly 0.1 to 0.2 gm. portions of the sodium carbonate, dissolve in about 50 c.c. of water in an Erlenmeyer flask, and titrate using methyl orange as an indicator. One hundred c.c. of N/10 acid are equivalent to 0.530 gm. of dried sodium carbonate.

[1] Hulett and Bonner: *J. Am. Chem. Soc.*, 31, 390, 1909.

fore, in order to express the total acidity of the 24-hour urine specimen in equivalent grams of sodium hydroxide, multiply the value of x, as just determined, by 0.004, or multiply the value of x by 0.0063 if it is desired to express the total acidity in grams of oxalic acid.

Interpretation.—(Under the heading "*Interpretation*" there will be found, in connection with the various quantitative methods which follow, brief notes as to the possible significance of the results obtained. For some further points (and reference to literature) see the chapters on the Normal and Pathological Constituents of Urine and on Studies in Metabolism. (For clinical interpretation see text-books on clinical diagnosis). The acidity of the urine expressed in cubic centimeters N/10 alkali required to neutralize the 24-hour output varies ordinarily from 200 to 500 under normal conditions with an average of perhaps 350. It is dependent almost entirely upon the diet, being low on a vegetable (base-forming) diet and high on a diet containing much meat, rice, whole wheat products, fruits containing benzoic acid, as prunes and cranberries, etc. (acid-forming foods). On the administration of 15 grams of sodium bicarbonate it may go down to 100; the ingestion of much acid-forming food may increase it to 600. In fasting it may rise in a few days to 800. It must be borne in mind that acidities of less than 250 usually indicate a true *alkalinity* of the urine inasmuch as phenolphthalein changes in an alkaline solution. Samples of urine collected shortly after a meal may be alkaline due to the so-called "alkaline tide."

Bacterial decomposition of the urea of the urine occurring in the urinary tract will increase the amount of ammonia and decrease the acidity of the urine. The same change usually occurs in urine left in contact with the air. The acidity of the urine is increased in acidosis, cardio-renal and certain other disorders. The acidity of the urine may be somewhat increased by administration of mineral acids, acid phosphates, or benzoates, but it is much more difficult to increase than to decrease this acidity.

Determination of Organic Acids. Method of Van Slyke and Palmer.[1] **Principle.**—Carbonates and phosphates are precipitated and the filtrate titrated with acid from the turning point for phenolphthalein to the turning point for tropaeolin OO. In diabetes this titration approximates closely the amount of beta-hydroxybutyric and acetoacetic acids in the urine and may be substituted for the determination of these substances for clinical purposes.

Procedure.—Mix 100 c.c. of urine with 2 gm. finely powdered calcium hydroxide and stir occasionally for 15 minutes. Filter. Carbonates and phos-

[1] Van Slyke and Palmer: *Jour. Biol. Chem.* 41, 567, 1920.

phates are removed. Transfer 25 c.c. of filtrate to a 125–150 c.c. test tube, add 0.5 c.c. 1 per cent phenolphthalein and 0.2 N HCl from a burette until pink color just disappears. This amount need not be measured. The pH is now about 8. Add 5 c.c. 0.02 per cent tropaeolin OO little by little with stirring to prevent precipitation of the dye. Titrate with 0.2 N HCl until the red color is that of a standard made from 0.6 c.c. 0.2 N HCl, 5 c.c. tropaeolin OO solution and water to make 60 c.c. When the end point is nearly reached dilute unknown to 60 c.c.

Calculation.—Subtract from c.c. 0.2 N HCl the amount (usually 0.7 c.c.) of the acid required to titrate a control tube of water between the same limits. Multiply the difference by 2 to get 0.1 N HCl and by 1000/25 to get the value for 1000 c.c. of urine.

Interpretation.—The titration includes organic acids, creatine, creatinine and a small amount of amino acids. Normally the excretion for 24 hours corresponds to 400–750 c.c. 0.1 N HCl or about 8 c.c. per kilo of body weight. In diabetic acidosis values from 20 to 180 c.c. per kilo have been observed.

Hydrogen Ion Concentration or True Acidity

Colorimetric Method. Principle.—The reaction of the urine is determined by matching the colors produced when pH indicators are added respectively to the diluted urine and to standard solutions of known reaction. This method is subject to salt and dilution errors but is sufficiently accurate for most clinical purposes.

Procedure.—Preparation of Standard Buffer Solutions: The Sørensen phosphate buffer standards, together with the Walpole acetate standards cover satisfactorily the range of pH of urine. The acetate buffers are prepared by mixing 0.1 N acetic acid and 0.1 N sodium acetate in the following proportions:

pH	C.c. 0.1 N acetic acid	C.c. 0.1 N sodium acetate
3.6	185	15
3.8	176	24
4.0	164	36
4.2	147	53
4.4	126	74
4.6	102	98
4.8	80	120
5.0	59	141
5.2	42	158
5.4	29	171
5.6	19	181

The phosphate buffers are prepared by mixing M/15 disodium phosphate and M/15 potassium acid phosphate in the following proportions:[1]

pH	C.c. M/15 Na_2HPO_4	C.c. M/15 KH_2PO_4
5.4	3.0	97.0
5.6	5.0	95.0
5.8	7.8	92.2
6.0	12.0	88.0
6.2	18.5	81.5
6.4	26.5	73.5
6.6	37.5	62.5
6.8	50.0	50.0
7.0	61.1	38.9
7.2	71.5	28.5
7.4	80.4	19.6
7.6	86.8	13.2
7.8	91.4	8.6
8.0	94.5	5.5

If it is desired to use the Clark and Lubs standard buffer mixtures[2] these may be prepared as described in Chapter I, page 13, *et seq.*

Preparation of Indicator Standards.—The selection of indicators depends on the pH range. See table on page 222. For urine, bromcresol green (pH 4.0–5.6), bromcresol purple (pH 5.4–7.0) and phenol red (pH 6.6–8.2) may be used. Methyl red (pH 4.4–6.0) may be substituted for bromcresol green, but is not as stable. Aqueous solutions of 0.04 per cent strength are used. 10 drops (or 0.5 c.c.) of these indicators are added to 10 c.c. portions of the standard buffer solutions.

Indicator	C.c. N/20 NaOH per decigram
Thymol blue	4.7
Bromphenol blue	3.3
Bromcresol green	3.2
Bromcresol purple	4.1
Bromthymol blue	3.5
Phenol red	6.3
Cresol red	5.8

The Clark and Lubs sulphonephthalein indicators are best prepared in stock solution from which the 0.04 per cent indicator solution is made by diluting 1 : 10.

[1] Interpolated from the data of Sørensen by Mr. Bernard L. Oser.
[2] Clark: The Determination of Hydrogen Ions, Baltimore, 1925.

For the preparation of these stock solutions 0.1 gm. of the dry powder is ground in an agate mortar with the following quantities (1.1 equivalents) of N/20 NaOH. When solution is complete dilute to 25 c.c. with water.[1]

Determination.—The urine is collected and kept under mineral oil. Into a test tube of the same diameter as those used to contain the indicator standards, introduce 8 c.c. of recently boiled distilled water and 10 drops (or 0.5 c.c.) of the bromcresol purple indicator solution. To this diluted indicator solution now add a layer of mineral oil and then introduce, without exposure to air, 2 c.c. of urine. Stir gently and match with indicator standards in a comparator block. A control tube containing the urine diluted 1 : 5 should be used in making the comparison, as described in the hydrogen ion determination of blood (see page 450).

If the urine is too acid or alkaline to come within the range of bromcresol purple, repeat using either bromcresol green or phenol red, as may be indicated.

Calculation.—The colorimetric reading made on the diluted urine at room temperature may be approximately corrected to give the actual pH at 38°C., by subtracting 0.2 pH.[2] The latter factor is, however, not constant. (See following method.)

Interpretation.—The H ion concentration of the urine is influenced by the same factors as the titratable acidity (see page 706). The normal pH values lie between 5.5 and 8.0 with a mean value of almost exactly 6.00. For vegetarians the mean value is about 6.64. In cardio-renal disorders the mean is 5.3. In most pathological conditions the hydrogen ion concentration is increased (pH decreased).

Bicolorimetric Methods.[3] **Principle.**—The bicolorimetric methods differ from the previous methods only in regard to the standards used. The bicolor standards of Hastings, Sendroy and Robson consist of a series of paired tubes containing the acid and alkaline forms of the indicators in the proportions corresponding to each pH as derived from the equation:

$$\mathrm{pH} = \mathrm{pK_1} + \log \frac{\text{alkaline form}}{\text{acid form}}$$

The indicators used are bromcresol green, chlorphenol red, bromcresol purple and phenol red. For details of preparing these standards the original paper must be consulted.

Myers and his associates have adopted the bicolorimeter (see page 363) to the determination of the pH of urine. The wedges are filled with acid and alkaline solutions of the proper indicators.

[1] These indicators may be obtained, dry or in prepared solutions, from the LaMotte Chemical Products Co. or Hynson, Westcott and Dunning, both of Baltimore, Maryland. Prepared and standardized buffer solutions may also be obtained.

[2] Muntwyler, Norris and Myers: Private communication.

[3] Hastings, Sendroy and Robson: *Jour. Biol. Chem.*, 65, 381, 1925.
Myers and Booher, *Proc. Soc. Exp. Biol. and Med.*, 22, 511, 1925.
Muntwyler, Norris and Myers, Private communication.

Procedure.—The procedure is the same as described on p. 709.

Calculations.—Hastings, Sendroy and Robson warm the diluted urine to 38° before reading, and correct only for the dilution by subtracting 0.1 pH. This gives results which are claimed to be within 0.05 pH of the electrometric pH of the undiluted urine at 38°.

Muntwyler, Norris and Myers read the diluted urine at room temperature, and correct for both temperature and dilution by subtracting 0.2 pH, to give the pH of the undiluted urine at 38°. They admit however that the correction factor is by no means constant. They claim that in view of the variability in salt content it is hardly possible that a single constant will hold accurately for all urine samples.

Electrometric Method.—The hydrogen ion concentration of urine may be determined by the titrimetric method of Meeker and Oser,[1] using quinhydrone electrodes, and diluted urine. The method is not applicable when the pH exceeds 8.0, or when the salt concentration is greater than fifth molar. The procedure is described in Chapter I, and the standard buffer solutions described under the colorimetric method for pH of urine (p. 708) are satisfactory.

Total Solids

1. **Drying Method.**—Place 5 c.c. of urine in a weighed shallow dish, acidify *very slightly* with acetic acid (1–3 drops), and dry it *in vacuo* in the presence of sulphuric acid to constant weight. Calculate the *percentage* of solids in the urine sample and the *total solids* for the 24-hour period.

Interpretation.—The average excretion of total solids by a normal adult man is about 70 grams. It is largely dependent upon the protein and salts of the diet. It may be decreased in severe nephritis due to impaired excretion, and greatly increased in diabetes with high sugar elimination.

Practically all the methods the technic of which includes evaporation at an increased temperature, either under atmospheric conditions or *in vacuo*, are attended with error.

Shackell's method[2] which entails the vacuum desiccation of the frozen sample is extremely satisfactory and should be used in all biological work where the greatest accuracy is desired.

2. **Calculation by Long's Coefficient.**—The quantity of solid material contained in the urine excreted for any 24-hour period may be approximately computed by multiplying the second and third decimal figures of the specific gravity by 2.6. This gives us the *number of grams of solid matter in 1 liter of urine.* From this value the total solids for the 24-hour period may easily be determined.

Calculation.—If the volume of urine for the 24 hours was 1120 c.c. and the specific gravity 1.018, the calculation would be as follows:

(*a*) $18 \times 2.6 = 46.8$ grams of solid matter in 1 liter of urine.

(*b*) $\frac{46.8 \times 1120}{1000} = 52.4$ grams of solid matter in 1120 c.c. of urine.

Long's coefficient was determined for urine, whose specific gravity was taken at 25°C. and is probably more accurate, for conditions obtaining in America, than the older coefficient of Haeser, 2.33.

Interpretation.—See above.

[1] Meeker and Oser: *Jour. Biol. Chem.*, 67, 397, 1926.
[2] Shackell: *American Journal of Physiology*, 24, 325, 1909.

Total Nitrogen

1. **Kjeldahl Method.**[1]—*Principle.*—The principle of this method is the conversion of the various nitrogenous bodies of the urine into ammonium sulphate by boiling with concentrated sulphuric acid, the subsequent decomposition of the ammonium sulphate by means of a fixed alkali (NaOH) and the collection of the liberated ammonia in an acid of known strength. Finally, this partly neutralized acid solution is titrated with an alkali of known strength and the nitrogen content of the urine under examination computed.

Procedure.—Place 5 c.c. of urine in a 500 c.c. long-necked Pyrex glass Kjeldahl flask, add 20 c.c. of concentrated sulphuric acid and about 0.2 gram of copper sulphate and boil the mixture for some time after it is colorless (about one hour). If a suitable hood or fume chamber is not available the sulphuric acid vapors may be carried away by suction. Connect the outlet tube of a 2–3 liter wash bottle filled with caustic soda solution with a suction pump. The inlet tube is connected with a Folin fume absorption tube such as illustrated in Fig. 221. If such a tube is not at hand a small funnel may be attached. The absorption tube is placed loosely over the mouth of the digestion flask and a constant current of air drawn through the apparatus.

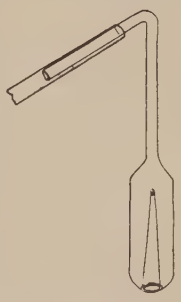


FIG. 221.—FOLIN FUME ABSORBER.

Allow the flask to cool and dilute the contents with about 200 c.c. of ammonia-free water. Add a little more of a concentrated solution of NaOH than is necessary to neutralize the sulphuric acid[2] and introduce into the flask a little coarse pumice stone or a few pieces of granulated zinc,[3] to prevent bumping, and a small piece of paraffin to lessen the tendency to froth. By means of a safety-tube connect the flask with a condenser so arranged that the delivery-tube passes into a vessel containing a known volume (the volume used depending upon the nitrogen content of the urine) of N/10 sulphuric acid, using care that the end of the delivery-tube reaches beneath the surface of the fluid.[4] Mix the contents of the distillation flask very thoroughly by shaking and distil the mixture until its volume has diminished about one-half. Titrate the partly neutralized N/10 sulphuric acid solution by means of N/10 sodium hydroxide, using Congo red as indicator, and calculate the content of nitrogen of the urine examined.

Calculation.—Subtract the number of cubic centimeters of N/10 sodium hydroxide used in the titration from the number of cubic centimeters of N/10 sulphuric acid taken. The remainder is equivalent to the number of cubic centi-

[1] There are numerous modifications of the original Kjeldahl method; the one described here, however, has given excellent satisfaction and is recommended for the determination of the nitrogen content of urine.

[2] This concentrated sodium hydroxide solution should be prepared in quantity and "check" tests made to determine the volume of the solution necessary to neutralize the volume (20 c.c.) of concentrated sulphuric acid used.

[3] Powdered zinc may be substituted.

[4] This delivery-tube should be of large caliber in order to avoid the "sucking back" of the fluid.

meters of N/10 sulphuric acid, neutralized by the ammonia of the urine. One c.c. of N/10 sulphuric acid is equivalent to 0.0014 gram of nitrogen. Therefore, if y represents the volume of urine used in the determination, and y′ the number of cubic centimeters of N/10 sulphuric acid neutralized by the ammonia of the urine, we have the following proportion:

$y : 100 :: y' \times 0.0014 : x$ (percentage of nitrogen in the urine examined).

Calculate the quantity of nitrogen in the 24-hour urine specimen.

Interpretation.—An adult of medium size on a mixed diet will usually excrete 12–18 grams of nitrogen per day. It varies, however, almost directly with the protein ingestion and hence usually runs parallel to the excretion of urea (see page 721). In a normal adult the total nitrogen of the feces and of the urine will often be almost exactly equal to the total nitrogen of the food. Such a condition is called "nitrogen equilibrium." The feces usually contain very little nitrogen. (See also Ammonia, Creatinine, etc.)

Calculation of Percentage Nitrogen Distribution.—In modern metabolism studies where the various forms of nitrogen are determined, in addition to the total nitrogen as yielded by the Kjeldahl method, it is customary to indicate what portion of the total nitrogen was present in the form of each of the individual nitrogenous constituents. These percentage values are secured by dividing the weight (grams) of nitrogen excreted for the day in the form of each individual nitrogenous constituent by the weight of the total nitrogen output for the same period. For example, if the total nitrogen excretion is 9.814 grams and the excretion of urea-nitrogen is 8.520 grams and the excretions of nitrogen in the forms of ammonia and creatinine are 0.271 gram and 0.639 gram respectively, the percentage distribution for these forms of nitrogen would be calculated as follows:

8.520 grams urea-nitrogen	÷ 9.814 grams total nitrogen	=	84.3 per cent
0.271 gram ammonia-nitrogen	÷ 9.814 grams total nitrogen	=	2.7 per cent
0.639 gram creatinine-nitrogen	÷ 9.814 grams total nitrogen	=	6.5 per cent

Nitrogen Partition in Urines Containing Albumin.—If the urine to be tested contains albumin this must be removed before an attempt at a nitrogen partition is made. This may be done by heating to boiling, acidifying with acetic acid to coagulate the protein, filtering and making up the filtrate to the original volume of the urine. If very small amounts of albumin are present this is attended with difficulty. In these cases Tracy and Welker[1] have suggested the use of aluminium hydroxide cream. It apparently removes none of the nitrogenous constituents of normal urine.

[1] Tracy and Welker: *Jour. Biol. Chem.*, 22, 55, 1915. For other applications of aluminum hydroxide precipitation of colloids, see Welker and Marshall, *J. Am. Chem. Soc.*, 35, 820, 1913.

Procedure.—One liter of urine (containing not over 1 per cent of albumin) is mixed with one liter of aluminum hydroxide cream[1] and filtered.

2. Folin-Wright Simplified Macro-Kjeldahl Method.[2]—*Principle.*—The method differs from the Kjeldahl procedure in that the digestion of the urine is brought about by means of a mixture of phosphoric and sulphuric acids aided by ferric chloride, and that the liberated ammonia is distilled without the use of a condenser. In this manner the time required for the completion of a determination is very much shortened.

Procedure.—Place 5 c.c. of urine in a 300 c.c. Kjeldahl flask (Pyrex), add 5 c.c. of phosphoric-sulphuric acid mixture,[3] 2 c.c. of 10 per cent ferric chloride solution, and 4 to 6 small pebbles or glass beads to prevent bumping. Boil vigorously in a hood over a microburner. The burner should give a strong flame and the top of the burner should be not more than 1 cm. away from the bottom of the flask. In 3 or 4 minutes the foam which forms at first will entirely disappear and the flask will be filled with dense white fumes. When this stage is reached (but no earlier) cover the mouth of the flask with a small watch glass and continue the vigorous heating for 2 minutes. At the end of 2 minutes dilute urines will be green or blue and concentrated urines will be a light straw yellow, the black carbonaceous matter being completely destroyed.[4] Turn the flame very low and continue the gentle boiling process for 2 minutes. Remove the flame, let the flask cool for 4 to 5 minutes (not longer), add 50 c.c. of ammonia free water, then 15 c.c. of saturated sodium hydroxide solution (50 to 55 per cent) and connect the flask promptly, by means of a rubber stopper and ordinary glass tubing, with a receiver containing from 35 to 75 c.c. of N/10 acid together with enough water to make a total volume of 150 c.c., and a drop or two of alizarin red. (The arrangement of the distillation apparatus is shown in Fig. 222). As soon as connection with the receiver is made, apply the flame again at full force, but not directly under the center of the flask until the acid and alkali have had time to mix. The contents of the flask begin to boil almost at once and 4 to 5 minutes boiling transfers all of the ammonia to the receiver. Under the conditions described the temperature of the contents of the receiver reaches only 65–70°C. Disconnect

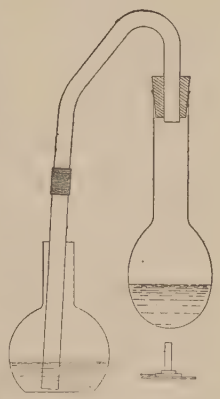


FIG. 222.—FOLIN WRIGHT DISTILLATION APPARATUS.[2]

[1] *Aluminum Hydroxide Cream.*—To a 1 per cent solution of ammonium alum at room temperature add a slight excess of a 1 per cent solution of ammonium hydroxide. Wash by decantation until the wash water shows only the faintest trace of residue on evaporation. Stronger solutions should not be used.

[2] Folin and Wright: *Jour. Biol. Chem.*, 38, 461, 1919.

[3] To 50 c.c. of 5 to 6 per cent copper sulphate solution add 300 c.c. of 85 per cent phosphoric acid and 100 c.c. of concentrated sulphuric acid.

[4] This method as far as the destructive digestion is concerned is primarily intended for urine only. It is not applicable to highly resistant materials, as for example milk, which cannot be completely destroyed in 6 minutes. Urines containing much sugar belong in this class. If 2 c.c. of fuming sulphuric acid are used in addition to 5 c.c. of the regular reagent sugar urines are readily destroyed within the required heating period of 4 to 5 minutes.

the receiver and titrate the excess of acid with N/10 alkali. If the distillate is titrated without cooling it is essential that a faint red color shall be accepted as the end point. The color will deepen on cooling and if time permits it is better to cool the distillate in running water before titrating.

Calculation.—Same as Kjeldahl method (p. 711).

Interpretation.—Same as Kjeldahl method (p. 712).

3. **Folin-Farmer Microchemical Method.**[1]—*Principle.*—This method belongs with the so-called microchemical methods inasmuch as it is adapted to the determination of amounts of nitrogen in the neighborhood of 1 mg. while in the ordinary Kjeldahl procedure 30–100 mg. of nitrogen are generally manipulated. One c.c. of diluted urine is decomposed with sulphuric acid as in the Kjeldahl method, the ammonia formed is set free by the addition of alkali and carried over into an acid solution by means of a current of air. The ammonia solution is then treated with Nessler's reagent and the color produced compared with that of a standard solution of an ammonium salt treated in the same way.

Procedure.—Introduce 5 c.c. of urine into a 50 c.c. volumetric flask if the specific gravity of the urine is over 1018, or into a 25 c.c. flask if the specific gravity is less than 1018.[2] Fill the flask to the mark with distilled water and invert it several times in order to guarantee thorough mixing. Transfer 1 c.c. of the diluted urine to a large (20–25 mm. × 200 mm.) Pyrex glass test-tube. Add to this 1 c.c. of concentrated sulphuric acid, 1 gram of potassium sulphate, 1 drop of 5 per cent copper sulphate solution and a small, clean, quartz pebble or glass bead. (The pebble or bead is added to prevent bumping.) Boil the mixture over a micro-burner for about six minutes, i.e., about two minutes after the mixture has become colorless. Allow to cool until the digestion mixture begins to become viscous. This ordinarily takes about three minutes, but in any event the mixture must not be permitted to solidify. Add about 6 c.c. of water (a few drops at a time, at first, then more rapidly) to prevent solidification. To this acid solution add an excess of sodium hydroxide (3 c.c. of a saturated solution is sufficient) and aspirate the liberated ammonia by means of a rapid air current[3] into a volumetric flask (100 c.c.) containing about 20 c.c. of ammonia-free water and 2 c.c. of N/10 hydrochloric acid (see Figs. 223 and 224, page 715). The air current should be only moderately rapid for the first two minutes but at the end of this two-minute period the current should be run at its maximum speed for an interval of eight minutes.

[1] Folin and Farmer: *Jour. Biol. Chem.*, 11, 493, 1912

[2] The purpose is to so dilute the urine that 1 c.c. of the diluted fluid shall contain 0.75–1.5 mg. of nitrogen.

[3] Either a vacuum pump or compressed air or a force pump may be used. The compressed air method is rather the more convenient inasmuch as the ammonia may be collected directly in a volumetric flask. Inasmuch as the necks of such flasks (100 c.c.) are not large enough to permit of the use of a two-hole rubber stopper when suction is used, the ammonia should be collected in one of the Pyrex test-tubes previously described which contains 2 c.c. of N/10 hydrochloric acid and about 5 c.c. of ammonia-free water. The ammonium salt is then transferred to the volumetric flask with 40–50 c.c. of water and nesslerized as described.

Disconnect the flask, dilute the contents to about 60 c.c. with ammonia-free water and dilute similarly 1 mg. of nitrogen in the form of ammonium sulphate[1] in a second volumetric flask. Nesslerize both solutions as nearly as possible at the same time with 5 c.c. of Nessler solution[2] diluted, immediately before using, with about 25 c.c. of ammonia-free water to avoid turbidity. Immediately fill the two flasks to the mark with ammonia-free water, mix well

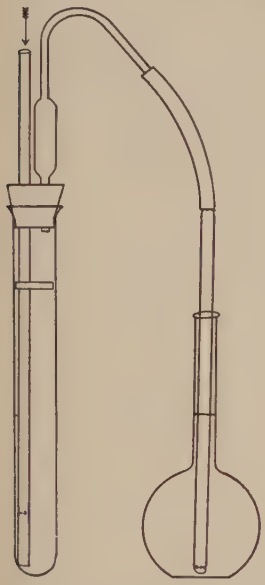


FIG. 223.

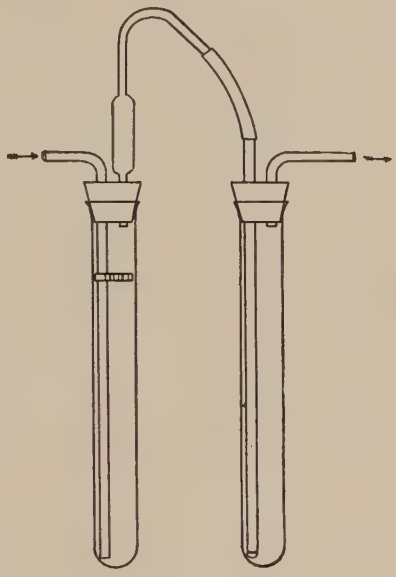


FIG. 224.

FIGS. 223 AND 224.—FORMS OF AERATION APPARATUS.
(*Folin and Farmer.*)

and determine the relative intensity of the two colors by means of a Duboscq colorimeter.[3]

The color of the unknown should be adjusted to that of the standard both from above and below the level of the latter. The matching of the colors is ordinarily very easy. It is desirable to make the readings by diffused daylight if possible. If electric light must be used, a sheet of smooth white paper should be interposed between the colorimeter and the source of light.

Calculation.—The reading of the standard divided by the reading of the unknown gives the nitrogen in milligrams in the volume of the urine taken. Calculate the total nitrogen output for the 24-hour period.

Interpretation.—See page 712.

[1] Care should be taken to secure the pure salt. All ammonium salts contain pyridine bases which titrate like ammonia but do not react with Nessler's reagent. *Pure* ammonium sulphate may be prepared by decomposing a high-grade ammonium salt with sodium hydroxide and passing the liberated ammonia into pure sulphuric acid. The salt is then precipitated by means of alcohol, then brought into solution in water and re-precipitated by alcohol. The final product should be dried in a desiccator over sulphuric acid. Pyridine-free ammonium salts are now obtainable on the market.

[2] See "Reagents and Solutions," Appendix.

[3] The standard may be set at any desired depth but a very satisfactory depth is 20 mm. The depth should be uniform throughout any series of comparative tests.

4. Bock and Benedict's Modification of the Folin-Farmer Procedure.—Bock and Benedict[1] have found distillation of the ammonia more accurate than aspiration. They connect the large Pyrex test-tube in which the digestion was carried out with a small Liebig condenser (made from a piece of glass tubing 30 by 150 mm. with two-hole rubber stoppers at each end through which pass the inlet and outlet tubes and the condenser tube itself). See Fig. 225. The lower end of the condenser is connected with a glass tube (or better an old pipette, to prevent back suction) which reaches nearly to the bottom of the volumetric flask used as a receiver. The distillation tube also has a two-hole rubber stopper. It is connected with the condenser and also carries a long straight tube which reaches nearly to the bottom of the test-tube, and is closed above with a piece of rubber tubing and a pinch-cock. The digestion is carried out just as in the Folin-Farmer method (see page 714) and when partially cool 7 c.c. of water

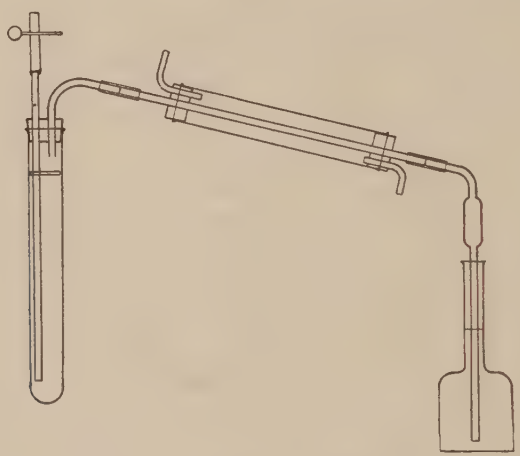


FIG. 225.—BOCK AND BENEDICT DISTILLATION APPARATUS.

are added. Into the long tube passing through the stopper suck 3 c.c. of saturated sodium hydroxide solution and close the pinch-cock. Insert the stopper, connect with the condenser and allow the alkali to run into the test-tube. The fluids are mixed by blowing a few bubbles of air through the apparatus. The test-tube is then heated to vigorous boiling (over a large free flame), the distillation being continued until a separation of salts occurs in the test-tube and the mixture begins to bump. This distillation requires about two minutes. The test-tube is then disconnected from the condenser and the latter washed down with a few cubic centimeters of water. The liquid in the receiving flask is diluted and nesslerized as in the Folin-Farmer method (see page 714).

Bock and Benedict, while holding the distillation procedure to be more accurate than aspiration, do not consider that the colorimetric method is equivalent to the standard Kjeldahl procedure in accuracy or reliability, although usually it agrees with the latter method within about 2–3 per cent, and is indispensable where very small amounts of

[1] Bock and Benedict: *Jour. Biol. Chem.*, 20, 47, 1915.

nitrogen are to be determined. According to Folin[1] and others the method is capable of greater accuracy than this, and the aspiration procedure gives satisfactory results. The method should be checked up carefully by each new learner of the method, using pure solutions. Outside air is better than laboratory air for aspiration purposes. Care is needed in using the pipettes, which should be of the Ostwald type and accurate. In using them allow the pipette to drain against the side of the vessel for 10 seconds and then blow out clean so that nothing is left behind in the tip. The reagents used must be as free as possible from ammonia and must be checked up, particularly the sulphuric acid and potassium sulphate. If desired, instead of using a colorimeter one may substitute titration with N/50 hydrochloric acid using alizarin, or better methyl red, as an indicator.

Rose[2] has described an apparatus combining aeration with distillation which, together with the use of perchloric acid and hydrogen peroxide in the digestion, yields a very accurate and rapid micromethod.

5. Direct Nesslerization Method of Koch and McMeekin.[3]—*Principle.*—The organic matter is destroyed by digestion with sulphuric acid assisted by hydrogen peroxide and the resulting solution nesslerized directly.

Procedure.—Dilute 5 c.c. of the well mixed urine to exactly 50 c.c. in a volumetric flask. If the specific gravity is over 1.018 dilute to 100 c.c. instead. The amount of any substance used for the determination should contain 0.3 to 1.0 mg. of nitrogen.

Measure 1 c.c. of diluted urine into a 20 × 2.5 cm. Pyrex test tube, add 1 c.c. of 1 :1 sulphuric acid[4] and heat over a free flame (or on a sand bath) with shak-

[1] Folin: *Jour. Biol. Chem.*, 21, 195, 1915.

[2] Rose, A. R.: *Jour. Biol. Chem.*, 64, 253, 1925.

[3] Koch and McMeekin: *J. Am. Chem. Soc.*, 46, 2066, 1924.

[4] Reagents: (*a*) *A 30% solution of hydrogen peroxide* (Merck's Superoxal or Kahlbaum's Perhydrol). The 30% hydrogen peroxide is a very reactive solution. Keep this in a cool place to avoid sudden decomposition. Do not allow the solution to come in contact with the skin and do not use a pipette to measure it with. The vapors are very irritating to the mucous membrane. The H_2O_2 preparation may contain nitrogen in appreciable amounts. In such cases a careful blank estimation must be made and the proper correction made in the calculations. (*b*) *A 1 : 1 solution of sulphuric acid;* to distilled water add an equal volume of concentrated c.p. sulphuric acid. (*c*) *The modified Nessler-Folin reagent:*

Dissolve 22.5 g. of iodine in 20 c.c. of water containing 30 g. of potassium iodide. After the solution is complete add 30 g. of pure metallic mercury, and shake the mixture well, keeping it from becoming *hot* by immersing in tap water from time to time. Continue this until the supernatant liquid has lost all of the yellow color due to iodine. Decant the supernatant aqueous solution and test a portion by adding a few drops thereof of 1 c.c. of a 1% soluble starch solution. Unless the starch test for iodine is obtained the solution may contain mercurous compounds. To the remaining solution add a few drops of an iodine solution of the same concentration as employed above, until a faint excess of free iodine can be detected by adding a few drops thereof to 1 c.c. of the starch solution. Dilute to 200 c.c. and mix well.

To 975 c.c. of an accurately prepared 10% sodium hydroxide solution now add the entire solution of potassium mercuric iodide prepared above. Mix thoroughly and allow to clear by standing.

This solution is to be used in the proportion of 10 c.c. per 100 c.c. of solution to be nesslerized, except in special cases where a great excess of acid is present, as in the direct

ing until the water has been removed. Then heat over a micro-burner until dense white fumes of sulphuric acid fill the tube. Let cool for about 30 seconds and then add by means of a medicine dropper one drop of 30 per cent hydrogen peroxide so that it falls directly into the mixture. Again boil gently for 2 to 5 minutes. If the discoloration returns repeat the treatment with hydrogen peroxide. (For more resistant materials than urine several drops of peroxide may be required at each step).

Allow the tube to cool and transfer quantitatively to a 100 c.c. volumetric flask diluting therein to make about 75 c.c. Now add 15 c.c. of the modified Nessler reagent, dilute at once to 100 c.c. and mix well. Prepare a standard by introducing into a 100 c.c. volumetric flask 1 c.c. of 1 :1 sulphuric acid, 1.5 to 5 c.c. of standard ammonium sulphate solution (5 c.c. = 1 mg. N) dilute to about 75 c.c. and nesslerize as above. Compare unknown with standard set usually at 20 or 30 mm.

Calculation.—$\frac{\text{Reading of Standard} \times \text{mg. of Nitrogen in Standard}}{\text{Unknown Reading}} = \text{mg. of}$ nitrogen in 1 c.c. of diluted urine added.

6. Direct Nesslerization Method of Folin and Denis.[1]—*Principle.*—A small amount of urine is digested with a mixture of sulphuric and phosphoric acids to destroy the organic matter, the digestion mixture treated directly with Nessler's reagent and compared with a standard ammonia solution also nesslerized.

Procedure.—This determination requires 0.7 to 1.5 mg. of nitrogen. Dilute 10 or 20 c.c. of urine to 100 c.c., mix and with an Ostwald pipette transfer 1 c.c. of the diluted urine to a large hard glass test tube. With an ordinary pipette add 1 c.c. of the phosphoric-sulphuric acid-copper sulphate mixture[2] together with a small pebble, to prevent bumping. Heat over a micro burner until the water is driven off and fumes become abundant within the tube. This should take place in about two minutes. When filled with fumes close the mouth of the test tube with a watch glass and continue the boiling at such a rate that the tube remains filled with fumes yet almost none escape. Within two minutes after the mouth of test tube was closed the contents should become clear, and bluish or light green.

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nesslerization methods. In such methods one should aim to add an amount of the reagent sufficient to ensure the same alkalinity in the unknowns as in the standards.

(*d*) *Standard ammonium sulphate solution* in 0.05 N sulphuric acid (5 c.c. = 1 mg. N).

A factor which must be carefully controlled is the volume of Nessler reagent added, because the variation in alkalinity affects the intensity of the color. It requires approximately 8.3 c.c. of the modified Nessler reagent (containing 8.4% of sodium hydroxide) to neutralize the acid used in the digestion. If now an additional 6.7 c.c. of Nessler reagent is added for every 100 c.c. of final volume nesslerized, very nearly the same alkalinity will be obtained in every dilution, that is, a titratable alkalinity equivalent to about 0.56% sodium hydroxide. The most satisfactory results are obtained by always preparing the standards containing 1 c.c. of the 1:1 sulphuric acid in exactly the same volumes as the unknowns. By so doing the alkalinities and excess of reagent are so nearly identical that theoretical values are easily obtained. When the nesslerization is conducted in a 50 c.c. volume, 12 c.c. of the Nessler reagent should be used, and the standard should similarly be prepared in a 50 c.c. volume.

[1]Folin and Denis: *J. Biol. Chem.*, 26, 486, 1916.

[2]Made by mixing 50 c.c. of 5 per cent. copper sulphate solution with 300 c.c. of 85 per cent phosphoric acid and then adding 100 c.c. of concentrated sulphuric acid free from ammonia. Keep well covered.

Continue the gentle boiling for 30 to 60 seconds, longer, provided, however, that the total boiling period, with test tube closed, must not be less than two minutes. Remove the flame and let cool for a little less than two minutes, then add water. Rinse the hot digestion mixture (sometimes turbid from silica) into a 200 c.c. volumetric flask, using for this purpose about 125 c.c. of water.

Transfer 10 c.c. of standard ammonium sulphate solution containing 1 mg. of nitrogen into another 200 c.c. volumetric flask. Add 1 c.c. of the concentrated phosphoric-sulphuric acid mixture, to balance the acid in the unknown, and dilute to a volume of about 150 c.c. When both flasks are thus ready give each a whirl and add 30 c.c. of Nessler reagent. Shake a little more and dilute both flasks to the 200 c.c. mark.

If the unknown nesslerized digestion mixture is turbid, centrifuge a portion, giving a crystal clear fluid above a white sediment (silica). If the sediment is colored the nesslerization was not successful and the determination must be discarded. Compare unknown and standard in a colorimeter.

Calculation.—$\frac{\text{Reading Standard}}{\text{Reading of Unknown}}$ = mg. of nitrogen in amount of urine used. Calculate percentage of nitrogen and daily output in grams. It is advisable to conduct a blank determination on the reagents.

Urea

1. Urease Methods.—*Principle.*—These methods depend upon the principle that the enzyme *urease* is able, at ordinary temperatures, to transform urea, quickly and completely, into ammonium carbonate. Takeuchi[1] in 1909 discovered the presence of this enzyme in the soja or soy bean. The application of this enzyme to the determination of urea in urine, blood, etc., was first proposed by Marshall,[2] whose methods have been modified by Van Slyke and Cullen.[3] These latter investigators prepared a permanent preparation of the enzyme, in a water-soluble form, the use of which makes more convenient the rapid and accurate determination of urea in urine, blood and other biological fluids.

The urease method is probably the most satisfactory of all methods for the determination of urea. Other nitrogenous constituents such as allantoin are not decomposed by urease. The method involves no carefully regulated heating procedures, and is applicable to diabetic urines.

The procedure for the determination in urine consists in treating the urine sample with urease, aerating the ammonia formed into fiftieth-normal acid, and titrating the excess of acid with fiftieth-normal alkali. (For colorimetric procedure see page 722.)

[1] Takeuchi: *Journ. Coll. Agr.*, Tokyo, 1909, Part 1.

[2] Marshall, E. K., Jr.: *J. Biol. Chem.*, 14, 283, 1913; 15, 495, 1913; 15, 487, 1913; 17, 351, 1914.

[3] Van Slyke D. D., and Cullen, G. E.: *J. Am. Med. Ass'n* 62, 1558, 1914. See, also *J. Biol. Chem.*, 19, 141, 1914.

Preparation of Solid Urease.[1]—Digest one part of soy bean meal with five parts of water at room temperature, with occasional stirring, for an hour, and clear the solution by filtration through paper pulp or centrifugation. Pour this extract slowly, with stirring, into at least 10 volumes of acetone. The acetone dehydrates the enzyme preparation. Filter, dry in vacuum, and powder. The activity of the preparation is retained indefinitely. Thus prepared it is not perfectly soluble in water, but this fact interferes in no way with its use.

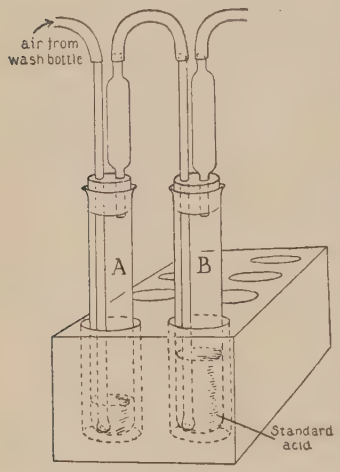


FIG. 226.—VAN SLYKE AND CULLEN AERATION APPARATUS.

Standardization of the Enzyme Preparation. Make up accurately a 3 per cent solution of pure urea. Treat this solution exactly as the urine is treated in the following method, using ½ c.c. of the solution. The ammonia formed should neutralize 25 c.c. of N/50 acid. If it does so the preparation is of sufficient strength to use as indicated. If not, more of the preparation must be used for a determination.

The ground soy bean may also be used directly in this determination. It should pass through a 20-mesh sieve. Rose and Coleman for their micro-procedure (see p. 722) use 0.2–0.4 gram of bean flour acting in a water-bath at 50–60° for five minutes. In their macro-method, using 5 c.c. of urine they dilute with 30 c.c. of water warmed to 50–60° and then add 5 grams of the soy bean flour and let stand for 30 minutes. They then add 5 c.c. of saturated sodium carbonate solution and aerate as usual.

(a) Procedure of Van Slyke and Cullen.—Dilute 5 c.c. of urine to 50 c.c. with ammonia-free water. Measure 5 c.c. of the diluted urine into Tube "A" (see Fig. 226), add 1 drop of caprylic alcohol (to prevent frothing), and 1 c.c. of enzyme solution.[2] Close "A" with stopper shown in figure, and let the tube stand 15 minutes for the enzyme to act. Measure into Tube "B" 25 c.c. of N/50 HCl or H_2SO_4. Add 1 drop of caprylic alcohol and 1 drop of a 1 per cent alizarin solution,[3] as indicator. Connect "A" and "B" as shown in the figure. At the end of 15 minutes aspirate for about one-half minute to remove any ammonia present in the free condition in "A." After this aspiration, open "A" and introduce 5 c.c. of saturated potassium carbonate. Close "A" at once and aspirate until all the ammonia has been removed from "A" and carried over into the acid in "B." The time needed for the aspiration varies for different pumps from 5 to 30 minutes, and should be determined by trial for the particular apparatus used. At the end of the time needed for the aeration,[4] the

[1] Van Slyke and Cullen: *Jour. Biol. Chem.*, 19, 211, 1914. Satisfactory preparations of urease in powder or tablet form may be obtained from the Arlington Chemical Company, Yonkers, N. Y., and from Hynson, Westcott and Dunning, Baltimore.

[2] The enzyme solution is prepared by dissolving 2 grams of the enzyme preparation, 0.6 gram of dipotassium-hydrogen phosphate, and 0.4 gram of monopotassium-dihydrogen phosphate in 10 c.c. of water. Solution is aided by stirring with a glass rod. The slightly opalescent solution should be covered with toluene and may be kept for two weeks without losing activity.

[3] Folin states that methyl red is preferable to alizarin for ammonia titrations.

[4] See Fiske (*Jour. Biol. Chem.*, 23, 455, 1915) and Van Slyke and Cullen (*Jour. Biol. Chem.*, 24, 117, 1916) for discussion of details of method.

pump is disconnected (care being taken to avoid back suction) and the excess acid in "B" is titrated by means of fiftieth-normal alkali.

Calculations.—The number of cubic centimeters of fiftieth-normal acid neutralized is multiplied by the factor 0.056 to give the number of grams of urea- plus ammonia-nitrogen in 100 c.c. of the urine. The ammonia alone may be determined at the same time as the ammonia plus urea, using the same technic except that 5 c.c. of the undiluted urine, no urease, and the factor 0.0056 are used for the determination of ammonia alone. The ammonia tubes are run in the same series as those for the urea determination, using the same air current for all.

Interpretation.—The mean average daily excretion of urea by normal adults is usually placed at about 25–35 grams but is very closely dependent upon the protein ingestion and hence may vary widely. It is of significance only when the amount of nitrogen ingested is known with some degree of accuracy. In disorders associated with increased tissue catabolism as in fevers, the excretion of urea is increased. It may be decreased in pronounced kidney and liver disorders due to decreased formation and decreased power of elimination, but these findings are not constant.

The per cent of the total nitrogen of the urine occurring as urea varies on the average from 80–90. On a high protein diet it is nearer 90 per cent; on a very low nitrogen but high calorie diet it may not be over 60 per cent. In marked acidosis it may be considerably decreased relative to the total nitrogen (see ammonia).

(b) Youngburg's Modification of Van Slyke and Cullen's Method.[1] —*Principle.*—The ammonia of the urine to be analyzed is removed by use of permutit, and urea is determined in the ammonia free filtrate by the Van Slyke and Cullen procedure using the alcoholic urease solution of Folin and Youngburg in place of the acetone insoluble urease.

Procedure.—Dilute 5 c.c. of urine to 50 c.c. (10 to 50 if very dilute urine) and mix well. Place 3 to 4 grams of dry permutit in a wide bottomed flask, preferable a 200 c.c. or 250 c.c. volumetric, and add 20 to 25 c.c. of the diluted urine. Agitate for 5 minutes. Allow to settle 15 to 30 seconds and pour through a thin filter paper which must not contain any appreciable amount of ammonia. If there is no permutit "dust" the urine may be decanted without filtering. To 5 c.c. of the ammonia free filtrate add 2 c.c. of alcoholic urease solution[2] and 2 drops of buffer solution.[3] Allow 15 minutes for decomposition of urea by the urease solution and proceed as in the Van Slyke and Cullen method.

[1] Youngburg, G. E.: *Jour. Biol. Chem.*, 45, 391, 1921.

[2] To prepare the alcoholic urease solution place 3 grams of permutit in a flask, wash once with 2 per cent acetic acid, then twice with water; and 5 grams of fine jack bean meal and 100 c.c. of 30 per cent alcohol. Shake gently but continuously for 10 to 15 minutes and filter. The filtrate contains practically all of the urease and extremely little of other materials.

[3] Dissolve and make up to 1000 c.c. 142 gms. Na_2HPO_4 and 120 gms. NaH_2PO_4 or equivalent amounts of the crystalline salts.

Calculation.—The number of cubic centimeters of fiftieth-normal acid neutralized by ammonia during aspiration multiplied by the factor 0.056 gives the number of grams of urea nitrogen in 100 c.c. of the original urine. In case the original dilution was 10 to 50 this value must be divided by 2.

Interpretation.—See page 721.

(*c*) *Colorimetric Modification of Van Slyke and Cullen's Method.*—Rose and Coleman[1] suggest the colorimetric determination of the ammonia which is carried over by the aspiration, rather than titration of the excess of acid. They nesslerize the solution in "B," and compare the color produced with the color of a nesslerized solution of known ammonia content, as in the Folin-Farmer method for total nitrogen. If this procedure is followed, the amount of urea and ammonia nitrogen in the solution acted upon by the urease must not exceed 2 mg. This procedure has been found useful where small quantities of urea are to be estimated.

(d) **Direct Nesslerization Method of Folin and Youngburg.**[2]—*Principle.*—The ammonia is removed from urine by permutit and direct nesslerization carried out on the ammonia—free filtrate following the decomposition of the urea by use of the permutit treated urease solution.

Procedure.—Place 1 c.c. of the ammonia—free urine filtrate (prepared as described in the preceding method) in a test tube, add 1 c.c. of the alcoholic urease solution, and 1 drop of buffer solution. Digest in a beaker of warm water (40–55°C.) for 5 minutes or at room temperature for 15 minutes, at the end of which time transfer the contents of the test tube to a 200 c.c. volumetric flask, diluting to a volume of about 150 c.c. Prepare a standard in another 200 c.c. flask by adding 1 mg. of N in the form of ammonium sulphate, 1 c.c. of urease solution, and enough ammonia free water to make about 150 c.c. Then add 20 c.c. of Nessler solution[3] to each flask, dilute to volume and compare the two solutions in a colorimeter.

Calculation.—The standard reading divided by the reading of the unknown gives the number of milligrams of urea N in 0.5 c.c. of undiluted urine.

Interpretation.—See page 721.

(e) **Marshall's Urease Method.**[4]—*Principle.*—This is a simple clinical method for the determination of urea in urine. It differs from the preceding method in that instead of aspirating off the ammonia formed from the urea by the action of the urease, it is titrated directly in the

[1] Rose and Coleman: *Biochem. Bull.*, 3, 411, 1914.
[2] Folin and Youngburg: *Jour. Biol. Chem.*, 38, 111, 1919; and Youngburg: *ibid.* 45, 319, 1921.
[3] See "Reagents and Solutions," Appendix.
[4] Marshall: *Jour. Biol. Chem.*, 14, 283, 1913.

urine mixture, thus simplifying the procedure. The method is nearly as accurate as the preceding, for normal urine the error being only about 2 per cent which is very satisfactory for a rapid clinical procedure. For diabetic urines the aeration procedure should be used as such urines contain substances which render the titration inaccurate.

Procedure.—Two 5 c.c. portions of the urine are measured into flasks of 200–300 c.c. capacity and diluted with distilled water to about 100–125 c.c. One c.c. of a 10 per cent solution of urease[1] prepared as described on page 720 is added to one flask, a few drops of toluene to each and the solution allowed to remain, well stoppered, at room temperature over night (or five hours). The fluid in each flask is titrated to a distinct pink color with N/10 hydrochloric acid using methyl orange as an indicator. A few cubic centimeters of the enzyme solution used should also be titrated to determine the amount of N/10 hydrochloric acid required to neutralize 1 c.c.

Calculation.—The amount of hydrochloric acid required for the contents of the flask containing the urine and enzyme solution, less the amount used for 5 c.c. of urine alone and that previously determined for 1 c.c. of enzyme solution, corresponds to the urea originally present in the sample of urine. Since 1 c.c. of N/10 HCl is equivalent to 3 mg. of urea, the number of cubic centimeters required, multiplied by 0.6 gives the value of urea expressed in grams per liter of urine.

Interpretation.—See page 721.

(f) Van Slyke's Manometric Method for Urea. Principle.—By action of urease the urea is converted to ammonium carbonate and the CO_2 in the latter estimated by manometric gas analysis. For procedure see p. 470.

(g) Stehle's Gasometric Method for Urea.[2]—*Principle.*—The urea is decomposed in a Van Slyke pipette by sodium hypobromite solution and the nitrogen measured.

Procedure.—Shake 25 c.c. of diluted urine (diluted 1:10) with 4 grams of permutit for 4 minutes. Filter. Introduce 1 c.c. of the filtrate into the Van Slyke blood gas apparatus, (see Fig. 130) rinsing in with 1 c.c. of water and 1 c.c. of sodium hydroxide solution.[3] Mix the contents of the apparatus and then introduce 1 c.c. of sodium hypobromite[3] solution. The mercury is lowered to the 50 c.c. mark and the apparatus is shaken vigorously for about half a minute. The aqueous solution is collected in the reservoir below the lower stop cock, mercury is admitted to the 50 c.c. chamber and, after adjusting the pressure, the volume of nitrogen is read.

Calculation.—This volume is corrected for the air dissolved in the urine and water, the air dissolved in the very strong alkali and hypobromite being negligible. The amounts of air measured at the temperature in question and at 760 mm. Hg. dissolved by 1 c.c. of water are as follows:

[1] In this particular method urease free from phosphate should be used as the presence of these salts interferes with the production of a satisfactory end-point.

[2] *Jour. Biol. Chem.*, 47, 13, 1921, and 51, 89, 1922.

[3] The sodium hydroxide solution is made by dissolving 100 g. of sodium hydroxide in 250 c.c. of water. To make the hypobromite solution dissolve 1 c.c. of bromine in 10 c.c. of the sodium hydroxide solution.

Temperature	Air in c.c.	Temperature	Air in c.c.
15	0.0216	22	0.0196
16	212	23	193
17	209	24	191
18	206	25	188
19	203	26	186
20	201	27	184
21	198	28	182

The corrected volume is then reduced to standard conditions using the formula

$$V_o = V \frac{B - W}{(1 + .00367\ t)760}$$

in which V_o is the volume under standard conditions, B the barometric reading, t the room temperature and W the vapor tension. The vapor tension of the solution over which the gas is collected is not that of pure water but of a strong hypobromite solution. Under the conditions of procedure here employed the tensions of the solution in the apparatus at various temperatures are as follows:

Temperature, °C.	W mm. Hg.	Temperature, °C.	W mm. Hg.
4	1.1	20	8.6
8	1.8	24	12.8
12	3.3	28	18.0
16	5.3		

In order to determine the nitrogen in milligrams one multiplies the volume, reduced to standard conditions, by 1.25, the weight of 1 c.c. of nitrogen.

(h) Wearn and Richards Method for Estimation of Minute Amounts of Urea. — This method was used by the authors in estimating the glomerular urine of frogs. By it nitrogen when present in as small an amount as 0.0001–0.0002 mg. may be determined with reasonable accuracy (± 10 per cent). For original article see Wearn and Richards, *Jour. Biol. Chem.*, 66, 275, 1925.

Ammonia

1. Folin's Method.—*Principle.*—The ammonia of the urine is set free by the addition of an alkali and this ammonia is then carried over by an air current into a flask containing a measured amount of standard acid. The excess acid is then titrated. The necessity for distillation is avoided.

Procedure.—Place 25 c.c. of urine in an aerometer cylinder, 30–40 cm. in height (Fig. 227, p. 725), add about 1 gram of dry sodium carbonate and introduce some crude petroleum to prevent foaming. Insert into the neck of the cylinder a

rubber stopper provided with two perforations, into each of which passes a glass tube, one of which reaches below the surface of the liquid. The shorter tube (10 cm. in length) is connected with a calcium chloride tube filled with cotton, and this tube is in turn joined to a glass tube extending to the bottom of a 500 c.c.

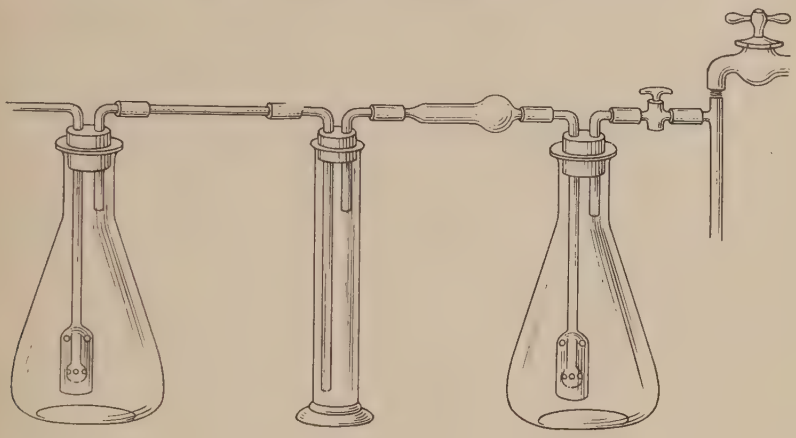


FIG. 227.—FOLIN AERATION APPARATUS.

wide-mouthed flask which is intended to absorb the ammonia and for this purpose should contain 20 c.c. of N/10 sulphuric acid, 200 c.c. of ammonia-free distilled water and a few drops of an indicator (alizarin red or Congo red). To insure the complete absorption of the ammonia the absorption flask is provided with a Folin improved absorption tube (Fig. 228), which is very effective in causing the air passing from the cylinder to come into intimate contact with the acid in the absorption flask. In order to exclude any error due to the presence of ammonia in the air a similar absorption apparatus to the one just described is attached to the other side of the aerometer cylinder, thus insuring the passage of ammonia-free air into the cylinder. With an ordinary filter pump and good water pressure the last trace of ammonia should be removed from the cylinder in about one and one-half hours.[1] The number of cubic centimeters of the N/10 sulphuric acid neutralized by the ammonia of the urine may be determined by direct titration with N/10 sodium hydroxide.

FIG. 228.—FOLIN IMPROVED ABSORPTION TUBE.

Steele[2] has suggested a modification for use on urines containing triple phosphate sediments. In this modification 0.5–1.0 gram of NaOH and about 15 grams of NaCl are substituted for the Na_2CO_3 of the Folin method. The use of sodium hydroxide and chloride instead of car-

[1] With any given filter pump a "check" test should be made with urine or, better, with a solution of an ammonium salt of known strength to determine how long the air current must be maintained to remove all the ammonia from 25 c.c. of the solution.

[2] Steele: *Jour. Biol. Chem.*, 8, 365, 1910.

bonate has also been recommended by other workers[1] as a general procedure, inasmuch as triple phosphate crystals are almost always formed on adding sodium carbonate and these are decomposed with some difficulty by sodium carbonate but readily by the hydroxide. It has not been shown that the use of sodium hydroxide in this manner brings about the decomposition of any other urinary nitrogen compounds.

Calculation.—Subtract the number of cubic centimeters of N/10 sodium hydroxide used in the titration from the number of cubic centimeters of N/10 sulphuric acid taken. The remainder is the number of cubic centimeters of N/10 sulphuric acid neutralized by the NH_3 of the urine. One c.c. of N/10 sulphuric acid is equivalent to 0.0017 gram of NH_3. Therefore if y represents the volume of urine used in the determination and y′ the number of cubic centimeters of N/10 sulphuric acid neutralized by the NH_3 of the urine, we have the following proportion:

$y : 100 :: y' \times 0.0017 : x$ (percentage of NH_3 in the urine examined).

Calculate the quantity of NH_3 in the 24-hour urine specimen.

Interpretation.—The average daily output of ammonia in the urine is about 0.7 gram, amounting to 2.5–4.5 per cent of the total nitrogen excretion. It is increased by the ingestion of acids or acid-forming foods and decreased by the ingestion of alkalis or base-forming foods. In acidosis it may be very greatly increased, being excreted in combination with hydroxybutyric and other acids. Values of 5 grams have been noted. It is at the same time increased relative to total nitrogen and urea. In pronounced liver disorders the same thing is noted, as ammonia is not so completely transformed into urea before excretion.

2. Micro-chemical Method of Folin and MacCallum.[2]—*Principle.*—This method is a combination of the aeration procedure for ammonia with its colorimetric determination by means of Nessler-Folin solution. It gives satisfactory results, but is probably not as accurate as the regular Folin procedure where the amount of substance for analysis is not limited.

Procedure.—By means of Ostwald pipettes introduce 1–5 c.c. of urine[3] into a Pyrex test-tube (20–25 by 200 mm.) and add to the urine a few drops of a solution containing 10 per cent of potassium carbonate and 15 per cent of potassium oxalate. To prevent foaming add a few drops of kerosene or heavy, crude machine oil. Pass a strong air current (see page 725) through the mixture

[1] Benedict and Osterberg: *Biochem. Bull.*, 3, 41, 1913.
Shulansky and Gies: *Biochem. Bull.*, 3, 45, 1913.

[2] Folin and MacCallum: *Jour. Biol. Chem.*, 11, 523, 1912.

[3] The volume of urine taken should contain 0.75–1.5 mg. of ammonia nitrogen. With normal urines 2 c.c. will generally yield the desired amount. With very dilute urines 5 c.c. may be required, while with diabetic urines rich in ammonium salts 1 c.c. may be excessive, thus requiring dilution.

until the ammonia has been entirely removed.[1] Collect the ammonia in a 100 c.c. volumetric flask containing about 20 c.c. of ammonia-free water and 2 c.c. of N/10 acid.

Nesslerize as described in the method for total nitrogen, page 715, and compare with 1 mg. of nitrogen obtained from a standard ammonium sulphate solution and similarly nesslerized.

It has been noted that a trace of something capable of giving a color with the Nessler-Folin solution continues to come long after all the ammonia has been removed from the urine. The nature of this substance has not yet been determined. In actual determinations by this method, the influence of this unknown substance, because of the small volume of urine used, is entirely negligible.

3. Formol Titration Method (Malfatti).[2]—*Principle.*—This method is based on the reaction taking place when formalin solution is added to a solution containing ammonium salts (see Amino-acid Nitrogen, below). An acid reaction is produced in the mixture, which is then titrated with standard alkali using phenolphthalein as an indicator. Amino-acids give the same reaction so that the result of the titration represents ammonia + amino-acid nitrogen. This method may be used for the rapid clinical estimation of these forms of nitrogen as a substitute for an ammonia determination, but the results do not represent ammonia as is sometimes stated.

Procedure.—To 25 c.c. of urine in a 200 c.c. Erlenmeyer flask add 15–20 grams of finely pulverized potassium oxalate, a few drops of phenolphthalein, and titrate to a faint but permanent pink color with N/10 NaOH. (The urine mixture just after neutralization in the urinary acidity determination (see page 705) may be used.) Then add 10 c.c. of neutral formalin solution (see amino-acid nitrogen), mix well and titrate with N/10 sodium hydroxide to a permanent pink color.

Calculation.—One c.c. of N/10 sodium hydroxide is equivalent to 1.7 mg. of ammonia. Multiply the number of cubic centimeters of N/10 alkali used by 1.7 and by 4 to get the number of milligrams of ammonia + amino-acid nitrogen (expressed as ammonia) in 100 c.c. of the urine examined.

4. Permutit Method.[3]—*Principle.*—The urine is shaken with particles of an "exchange silicate," which remove the ammonia from solution. The ammonia is set free from the silicate by treating with alkali solution. This is then nesslerized and compared with a standard ammonia solution treated in the same way.

Procedure.—Introduce about 2 gm. of permutit powder into a 200 c.c. volumetric flask. Add about 5 c.c. of water (no more), and with an Ostwald pipette introduce 1 or 2 c.c. of urine, or with a 5 c.c. pipette introduce 5 c.c. of previously

[1] Ordinarily a period of ten minutes is sufficiently long.
[2] Malfatti: *Z. anal. Chem.*, 47, 273, 1908.
[3] Folin and Bell: *Jour. Biol. Chem.*, 29, 329, 1917.

diluted urine (corresponding to 1 or 2 c.c. of the original urine). With urines very low in ammonia it may be necessary to use more urine (5 c.c.), but, in so far as it is practicable, it is better not to use more than 2 c.c. and to employ weaker standard (0.5 mg. of ammonia nitrogen) for the color comparison. Rinse down the added urine by means of a little water (1 to 5 c.c.), and shake gently but continuously for 5 minutes. Rinse the powder to the bottom of the flask by the addition of water (25 to 40 c.c.) and decant. Add water once more and decant. (In the case of urines rich in bile it is advisable to wash once or twice more.) Add a little water to the powder, introduce 2 c.c. of 10 per cent sodium hydroxide, shake for a few moments and set aside, while preparing the standard ammonium sulphate solution as follows:

Transfer 10 c.c. of the standard ammonium sulphate solution (see p. 715) containing 1 mg. of nitrogen to another 200 c.c. volumetric flask and add 2 c.c. of 10 per cent sodium hydroxide (to balance the alkali added to the permutit mixture in the other flask). Dilute to about 150 c.c. and mix. Transfer 20 c.c. of Nessler's solution (see p. 717) to a measuring cylinder. Now give the volumetric flask a vigorous whirl so as to set the solution spinning within the flask and add at once the whole of the Nessler solution in the cylinder. With another whirling movement complete the mixing of the contents of the flask. If the process of nesslerization has been successful a deep red but crystal clear solution is obtained. If it is not perfectly clear throw it away and prepare a fresh standard. Then dilute the contents of the flask containing the permutit and the urinary ammonia to about 150 c.c., whirl the mixture and add the Nessler reagent (20 c.c.) exactly as in the case of the standard solution. Dilute the contents of both flasks to volume (200 c.c.) and compare in a colorimeter with the standard set at 20 mm.

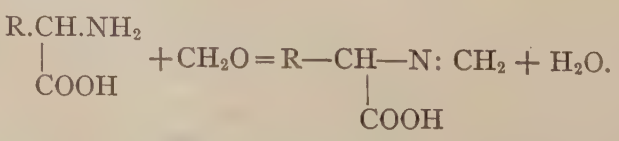
Calculation: $\frac{20 \text{ (reading of standard)}}{R \text{ (reading of unknown)}}$ = mg. of ammonia N in amount of urine used.

Calculate the per cent of ammonia and the 24 hour output.

Interpretation.—See page 726.

Amino-acid Nitrogen

1. Henriques-Sørensen Formol Titration Method.[1]—*Principle.*—A solution containing amino-acids is nearly neutral in reaction. If formaldehyde be added, however, the following reaction takes place with the formation of methylene derivatives which are more strongly acid in reaction due to the destruction of the basic properties of the amino groups. The carboxyl groups may then be titrated using phenolphthalein as an indicator.



[1] Henriques and Sørensen: *Zeit. physiol. chem.*, 64, 120, 1900.

The acidity as shown by the titration is a measure of the amount of amino-acid nitrogen present. Ammonia likewise reacts with formaldehyde in a similar manner as is shown in the following equation:

$$4NH_4Cl + 6CH_2O = N_4(CH_2)_6 + 6H_2O + 4HCl.$$

Hence the formol titration in the presence of ammonia gives results which include both amino-acid and ammonia nitrogen. Ammonia may be determined and a correction applied, or the ammonia may be removed by means of phosphotungstic acid. Phosphates also interfere by obscuring the end-point and are removed by the addition of barium salts.

It must be borne in mind that polypeptides and still more complex protein derivatives likewise react with formol to a certain degree so that the results do not strictly represent "amino-acid nitrogen."

The method is, with some modifications involving the preparation of the solution to be titrated, applicable in the determination of amino-acids in any medium, *e.g.*, urine, protein digests, etc. When poorly dissociated acids, *e.g.*, some fatty acids, are present, these will in part be included in the result and lead to values which are too high. Certain of the amino-acids when present in large amounts will give erroneous results, but in the ordinary urine or digest these errors are either negligible or compensate each other. In the titration of colored solutions the control solution which is necessary in this method must be colored to correspond with the color of the unknown solution.

Procedure.—The determination of the amino-acids is carried out as follows: The solution to be analyzed, if carbonates, phosphates and ammonia are absent, is made neutral to litmus (paper) and the solution titrated with formaldehyde as below.[1] In case carbonates, phosphates or ammonia are present a preliminary treatment is necessary which will vary according to the quantity of ammonia present.

(a) For Small Amounts of Ammonia.—Applicable to most urines. Fifty c.c. of the material under examination is pipetted into a 100 c.c. measuring flask and 1 c.c. phenolphthalein solution[2] and 2 grams of solid barium chloride are added; the whole is shaken, to saturate the solution with barium chloride; saturated barium hydroxide solution is added until the red color of the phenolphthalein develops and then an excess of 5 c.c. is added. The flask is filled to the graduation mark with water, shaken and permitted to stand for 15 minutes, after which it is filtered through a dry filter. Eighty c.c. of the clear red filtrate (which corresponds to 40 c.c. of the liquid under examination) are placed in a 100 c.c. measuring flask, neutralized to litmus and diluted to 100 c.c. with freshly boiled water. Equal portions of this solution, 40 c.c. (equivalent to

[1] As a standard of comparison the litmus paper used for neutralization is contrasted with a similar piece dipped in a phosphate solution having a neutral reaction (M/15 KH_2PO_4 and M/15 Na_2HPO_4).

[2] A solution of 0.5 gram of phenolphthalein in 50 c.c. of alcohol and 50 c.c. of water.

16 c.c. of the original solution), may be taken for analysis, one for the formol titration and the other for the determination of ammonia nitrogen.[1]

(b) For Large Amounts of Ammonia.—After the treatment with phenolphthalein, barium chloride, and barium hydroxide, and the solution has been diluted to 100 c.c. as in (a) above, the ammonia is distilled off, in vacuo.[2]

In case the solution is deeply colored, as in protein digests, it may be necessary to decolorize[3] before the titration is attempted.

Final Titration.—For the final titration a volume of from 20–40 c.c. which contains approximately 0.025 gram of nitrogen is the most desirable. A control solution is run composed of an equal volume of boiled distilled water and 20 c.c. of the formaldehyde mixture.[4] This control solution is colored[5] so that its tint matches that of the solution to be titrated.

To this control is added about half the volume of N/5 alkali which will be used in the titration of the solution under investigation and it is then titrated with N/5 acid to a faint red (first stage).[6]

An additional drop of N/5 alkali is added, which imparts a distinct red to the solution (second stage).

The solution to be analyzed is now titrated to the color produced in the second stage of the control. The formaldehyde mixture is now added; 10 c.c. for each 20 c.c. of the solution, and the mixture again titrated to the second stage with N/5 alkali.[7]

Two drops of the N/5 alkali are now added to the control solution which assumes a deep red color (third stage). Fifth normal alkali is now added to the solution under examination until it assumes a color corresponding to the third stage of the control. This completes the titration.

Calculation.—The calculations are similar to those which pertain to any acidimetry procedure. Each cubic centimeter of an N/5 alkali or acid solution is equivalent to 0.0028 gram of nitrogen. An example will illustrate the procedure: 40 c.c. of solution (16 c.c. of urine) required 5.10 c.c. N/5 NaOH; control, 0.10 c.c. N/5 NaOH; total required for amino-acids 5.00 c.c. equivalent to 0.014 gram of nitrogen. Ammonia nitrogen in 16 c.c. of urine 0.007 gram N. Then 0.014 − 0.007 = 0.007 gram amino-acid nitrogen in 16 c.c. of urine.

Interpretation.—The excretion of total amino-acid nitrogen by a normal adult averages between 0.4 to 1.0 gram per day or from 2 to 6 per cent of the total nitrogen. Free amino-acid nitrogen (see Van

[1] The determination of ammonia may be dispensed with in case a separate determination is made.

[2] For particulars with regard to the distillation, etc., see Henriques and Sørensen: *Zeit. physiol. Chem.*, 64, 137, 1909.

[3] For methods see Jessen-Hansen, Abderhalden's Arbeitsmethoden, vol. 6, p. 262, 1912.

[4] The formaldehyde solution is freshly prepared for each set of determinations as follows: to 50 c.c. of commercial formaldehyde (formol) (30–40 per cent) add 1 c.c. of the phenolphthalein solution. N/5 alkali is then added until the mixture acquires a faint red color. The volume of the formaldehyde used will vary with the volume of the solution to be analyzed; approximately 10 c.c. of the formalin solution are added for each 20 c.c. of the unknown solution.

[5] Solution of Bismark brown is very satisfactory for urines. Tropæolin O, tropæolin OO, *p*-nitro-phenol, methyl orange or alizarin sulphonate, may be used.

[6] This procedure is recommended in order that the final volume of the control and the unknown solutions shall be approximately the same when the process is complete.

[7] This is best accomplished by adding alkali until the color is deeper than that of the control, then acid again until lighter and finally alkali to the desired color.

Slyke procedure) is considerably less than this, ordinarily 0.5 to 1.0 per cent of the total nitrogen. The amount may be largely increased in disorders associated with tissue waste as typhoid, in pronounced atrophy of the liver, acidosis, etc.

2. Colorimetric Method of Folin.[1]—**Principle.**—The urine is treated with permutit to remove ammonia and then with naphthaquinone sulphonic acid which gives a red color. This color is compared with that obtained from a standard amino-acid solution.

Procedure.—Dilute from 5 to 25 c.c. of urine to a volume of 25 c.c. in a 50 c.c. Erlenmeyer flask. Add 2 to 3 grams of permutit and agitate very gently but continuously for 5 minutes. Decant the supernatant urine into another 50 c.c. flask. Add again 2 to 3 grams of permitut and shake as before for 5 minutes. By this double extraction with permutit every trace of ammonia is removed. Decant the supernatant urine into a flask or test tube. It may be a little turbid, but this fact does not interfere with the determination. To test tubes graduated at 25 c.c. add 1, 2, and 3 c.c., respectively, of a standard glycocoll solution in 0.1 N hydrochloric acid plus 0.2 per cent of sodium benzoate. This standard solution should contain 0.1 mg. of glycocoll nitrogen per c.c. To these tubes add 1, 2, and 3 c.c. respectively, of special 1 per cent sodium carbonate solution.[2] (One c.c. of sodium carbonate for each c.c. of 0.1 N hydrochloric acid present.) Dilute the contents of each test tube to a volume of 10 c.c.

Transfer 5 c.c. of ammonia-free (usually diluted) urine to another test tube graduated at 25 c.c. Add 1 c.c. of 0.1 N hydrochloric acid and 1 c.c. of the 1 per cent sodium carbonate solution. Dilute to 10 c.c. Dissolve 250 mg. of the amino-acid reagent (β-naphthaquinone monosodium sulphonate) in 50 c.c. of water, and add 5 c.c. of this solution to each standard and to the unknown urine.

Mix and set in a dark place over night. It is often advisable to take out the test tubes and inspect them after they have stood 10 to 15 minutes. If the test tube containing the urine appears much darker than the darkest standard, as may happen, especially in connection with experiments planned to produce excessive amino-acid excretion, then it is, of course, necessary to start another sample of the urine, taking only 1, 2, or 3 c.c. and treating it in the same way as the first sample, not omitting to provide for a final volume of 15 c.c.

The following day the standard and the unknown or unknowns are first acidified by the addition of 1 c.c. of the special 25 per cent acetic acid-acetate solution. To each are then added 5 c.c. of the 4 per cent sodium thiosulfate solution. The contents of all the tubes are diluted to a volume of 25 c.c. and,

[1] Folin: *Jour. Biol. Chem.*, 51, 393, 1922.

[2] Reagents Required for the Colorimetric Method.—(*a*) *Standard Amino-acid Solution.*—A solution containing 0.07 mg. nitrogen per c.c. Glycocoll may be used or leucine, phenylalanine or tyrosine.

(*b*) *Special Sodium Carbonate Solution.*—Fifty c.c. of approximately saturated solution are diluted to 500 c.c. Titrate 20 c.c. of 0.1 N hydrochloric acid with the carbonate, using methyl red as an indicator. Dilute so that 8.5 c.c. are equivalent to 20 c.c. of 0.1 N acid. The carbonate solution is about 1 per cent.

(*c*) *Amino-acid Reagent.*—A 0.5 per cent solution of the sodium salt of β-naphthaquinone sulphonic acid freshly prepared. The quinone itself may be prepared according to a method described in the original paper.

(*d*) *Special Acetic acid-Acetate Solution.*—Dilute 100 c.c. of 50 per cent acetic acid with an equal volume of 5 per cent sodium acetate solution.

(*e*) *Sodium Thiosulphate Solution.*—A 4 per cent solution of the crystalline salt.

after mixing, the color of the unknown is read against that of the standard having most nearly the same intensity of color.

For the calculation it is, of course, essential to know which standard is used, and the actual volume of undiluted urine taken for the determination.

Creatinine

Folin's Colorimetric Method.—*Principle.*—This method is based upon the characteristic property possessed by creatinine, of yielding a certain definite color-reaction in the presence of picric acid in alkaline solution. This reaction (Jaffe reaction) is due to the formation of a red tautomer of creatinine picrate.[1] The production of this tautomer is "dependent upon the formation of a salt, a keto-enol change within the creatinine molecule, and a change in the picric acid molecule involving the hydrogens in the *meta* positions and probably, all three nitro groups."[2]

Procedure.—Place 10 c.c. of urine in a 500 c.c. volumetric flask, add 15 c.c. of a saturated solution of picric acid and 5 c.c. of a 10 per cent solution of sodium hydroxide, shake thoroughly and allow the mixture to stand for five minutes. During this interval pour a little N/2 potassium bichromate solution[3] into each of the two cylinders of the colorimeter (Duboscq's, see Fig. 110, p. 361) and carefully adjust the depth of the solution in one of the cylinders to the 8 mm. mark. A few preliminary colorimetric readings may now be made with the solution in the other cylinder, in order to insure greater accuracy in the subsequent examination of the solution of unknown strength. Obviously the two solutions of potassium bichromate are identical in color and in their examination no two readings should differ more than 0.1–0.2 mm. from the true value (8 mm.). Four or more readings should be made in each case and an average taken of all of them exclusive of the first reading, which is apt to be less accurate than the succeeding readings. In time as one becomes proficient in the technic it is perfectly safe to take the average of the first two readings.

At the end of the five-minute interval already mentioned, the contents of the 500 c.c. flask are diluted to the 500 c.c. mark, the bichromate solution is thoroughly rinsed out of one of the cylinders and replaced with the solution thus prepared and a number of colorimetric readings are immediately made.

Ordinarily 10 c.c. of urine is used in the determination by this method, but if the content of creatinine is above 15 mg. or below 5 mg. the determination should be repeated with a volume of urine selected according to the content of creatinine. This variation in the volume of urine according to the content of creatinine is quite essential, since the method loses in accuracy when more than 15 mg. or less than 5 mg. of creatinine is present in the solution of unknown strength.

Calculation.—By experiment it has been determined that 10 mg. of pure creatinine, when brought into solution and diluted to 500 c.c. as explained in the above method, yields a mixture 8.1 mm. of which possesses the same colorimetric value as 8 mm. of a N/2 solution of potassium bichromate. Bearing this in mind the

[1] Greenwald and Gross: *Jour. Biol. Chem.*, 59, 601, 1924.

[2] Greenwald: *Jour. Am. Chem. Soc.*, 47, 1443, 1925.

[3] This solution contains 24.55 grams of potassium bichromate to the liter. A pure creatinine standard is to be preferred, see p. 733.

computation is readily made by means of the following proportion in which y represents the number of millimeters of the solution of unknown strength equivalent to the 8 mm. of the potassium bichromate solution:

y:8.1::10:x (mg. of creatinine in the quantity of urine used).

This proportion may be used for the calculation no matter what volume of urine (5, 10, or 15 c.c.) is used in the determination. The 10 represents 10 mg. of creatinine which gives a color equal to 8.1 mm., whether dissolved in 5, 10, or 15 c.c. of fluid.

Calculate the quantity of creatinine in the 24-hour urine specimen.

Interpretation.—The daily excretion of creatinine by an adult of medium weight averages about 1.25 grams. The value is nearly constant from day to day for a given individual being influenced by the diet hardly at all unless this contains much preformed creatinine (as in case of a heavy meat diet). The excretion of creatinine is to a certain extent a measure of muscular efficiency and of the amount of active muscle tissue in the body. Relative to body weight less creatinine is excreted by obese persons.

Creatinine excretion is decreased in disorders associated with muscular atrophy and muscular weakness. It increases with increased tissue catabolism as in fever.

By the "creatinine coefficient" is meant the number of milligrams of creatinine—*nitrogen* excreted daily per kilo of body weight. This varies under normal conditions from 7–11.

Use of Pure Creatinine Standards.—Instead of using as a standard a potassium dichromate solution as above indicated, a solution of pure creatinine is to be recommended. By using this certain arbitrary factors are eliminated and the method becomes of more general applicability. The standard need not be set at a definite mark as is necessary in the case of dichromate and temperature and time have less influence on the accuracy of the results. A stock solution of pure creatinine (made according to Benedict's directions; see Chapter XXVII on Physiological Constituents of Urine) is made by dissolving 1 gram of the substance in sufficient N/10 HCl to make a liter. This solution contains 1 mg. of creatinine per cubic centimeter. In carrying out the determination treat 10 c.c. of the stock solution in the same way and at the same time as the 10 c.c. sample of urine. Compare in the colorimeter. The calculation is simple. The reading of the standard divided by the reading of the urine gives directly the number of milligrams of creatinine per cubic centimeter of urine.

Folin's Microchemical Modification.[1]—*Principle.*—The principle is the same as that of the original colorimetric method (see page 732). This procedure is to be recommended particularly where only small amounts of material are available.

Procedure.—One c.c. of the standard creatinine (see above) solution (1 mg. per c.c.) is measured into a 100 c.c. volumetric flask and 1 c.c. of urine into another; 20 c.c. of saturated picric acid solution (measured with a cylinder) are added to each and then 1.5 c.c. of a 10 per cent solution of sodium hydroxide. At the end of ten minutes the flasks are filled up to the mark with tap water and the color

[1] Folin: *Jour. Biol. Chem.*, 17, 469, 1914.

of the unknown is determined. The reading of the standard divided by the reading of the unknown gives directly the number of milligrams of creatinine in the amount of urine taken for analysis.

3. Shaffer's Modification for the Determination of Creatinine in Very Dilute Solutions.[1]—The regular Folin procedure is not accurate when applied to urines containing less than 20 mg. of creatinine per 100 c.c. By a slight modification it becomes applicable to creatinine solutions containing as little as 1 mg. or less per 100 c.c.

Procedure.—To the solution under examination add an equal volume of saturated picric acid solution and one-tenth this volume of 10 per cent sodium hydroxide solution. After standing 6–10 minutes the liquid is diluted to a definite volume depending upon the intensity of the color developed. With very dilute solutions one may add solid picric acid equivalent to half saturation (0.6 per cent) and when dissolved, one-twentieth the volume of sodium hydroxide. Provided the creatinine solution itself has not sufficient color to interfere, the results by this method appear to be as accurate as the original procedure. The colorimetric readings and calculations are made in the same way as in the preceding methods.

Creatine

1. Folin-Benedict Method.[2]—*Principle.*—Creatine on boiling with acid is transformed into creatinine. By determining the content of creatinine before and after the acid treatment we are able to calculate the amount of creatine originally present in the urine. The Folin colorimetric method (page 732) is used for determining the creatinine in both cases. The method is not applicable to diabetic urines.

Procedure.—Introduce into a small flask or beaker 10 c.c. of the urine to be examined. (If 10 c.c. contains more than 12 or less than 7 mg. of total creatinine use a correspondingly smaller or larger volume of urine.) Add from 10–20 c.c. of normal HCl, and a pinch or two of powdered or granulated lead. Boil the mixture over a free flame as slowly or as rapidly as may be desired, until very nearly down to dryness, when the heating should be continued to dryness either on the water-bath or very easily by simply holding the vessel in the hand and heating carefully for a moment or two. Let the residue stand on the water-bath for a few minutes until most of the excess of hydrochloric acid gas has been expelled, after which dissolve it in about 10 c.c. of hot water and rinse the solution quantitatively through a plug of cotton or glass wool (to remove all metallic lead) into a 500 c.c. volumetric flask. Add 20–25 c.c. of a saturated picric acid solution and about 7–8 c.c. of a 10 per cent NaOH solution, which contains 5 per cent of Rochelle salt.[3] At the end of five minutes fill to the mark with water and read in the colorimeter just as in the case of creatinine (see page 732).

Calculation.—Calculate the creatinine content of the solution in the same manner as given under Creatinine (page 732). From the value thus obtained subtract the value for the creatinine content of the urine before dehydration. The difference will be the creatine content of the original urine in terms of creatinine.

[1] Shaffer: *Jour. Biol. Chem.*, 18, 525, 1914.
[2] Benedict: *Jour. Biol. Chem.*, 18, 191, 1914.
[3] The Rochelle salt should be present to prevent any formation of turbidity, which otherwise may occur, due to the presence of traces of dissolved lead.

Interpretation.—Creatine occurs only in very small amounts in the urine of normal adults, but is found in larger amounts in that of children (10 to 50 mg. per day). Creatine ingestion in adults has little effect on the urinary excretion. In fasting, the amount is markedly increased (it may amount to 100 mg. or more per day). Creatine also appears in the urine after high water ingestion. It is found in many pathological conditions associated with malnutrition and disintegration of muscular tissue, in fever, etc. Very large amounts have been found in cases of carcinoma of the liver.

2. Folin-Benedict and Myers Method.[1]—To 20 c.c. of urine in a 50 c.c. volumetric flask, add 20 c.c. of normal hydrochloric acid and place the flask in an autoclave at a temperature of 117–120°C. for one-half hour. Add distilled water until the volume of the acid-urine mixture is exactly 50 c.c., close the flask by means of a stopper, and shake it thoroughly. Approximately neutralize 25 c.c. of this mixture, introduce it into a 500 c.c. volumetric flask and determine its creatinine content according to Folin's colorimetric method (see page 732).

For calculation and interpretation see the foregoing method.

3. Method of Folin.[2]—*Water-bath Procedure.*—Heat 10 c.c. of urine with 5 c.c. of normal hydrochloric acid on the boiling water-bath or at 90°C. for three hours. The creatine is transformed into creatinine. Some darkening takes place but this does not interfere because of the subsequent dilution. The mixture is made up to 50 c.c., 25 c.c. of this is taken, neutralized, and creatinine plus creatine determined just as in the case of creatinine alone. The creatine is obtained by difference. This procedure may be used for diabetic urines which is not the case with the autoclave procedure nor with the Benedict modification. It is perhaps not quite so accurate as the autoclave procedure.

4. Microchemical Modification of Folin.[3]—By greatly diluting the urine the time required for the conversion of creatine to creatinine is decreased, and picric acid can be substituted for mineral acid.

Procedure.—Enough urine to give 0.7–1.5 mg. of creatinine is measured into a weighed Pyrex Erlenmeyer flask (capacity 200 c.c.); 20 c.c. of saturated picric acid solution, about 130 c.c. of water, and a few very small pebbles to promote even boiling are added and the mixture is gently boiled, preferably over a micro-burner for about one hour. At the end of this time the heat is increased and the solution is boiled down to rather less than 20 c.c. The flask is transferred to the scales and enough water is added to make the total solution equal to 20–25 grams. The solution is cooled in running water, 1.5 c.c. of 10 per cent sodium hydroxide are added, and the total creatinine is determined as in the preformed creatinine determination using 1 mg. of creatinine as a standard. The method has been found to give good results in the presence of glucose and other sugars.

Morris[4] has suggested that in the case of diabetic urines the total creatinine be determined after precipitation of the creatine and creatinine with picric acid. The method is not recommended as a regular procedure.

[1] Benedict and Myers: *Am. J. Phys.*, 18, 397, 1907.
[2] Folin: *Zeitschr. f. physiol. Chem.*, 41, 222, 1904.
[3] Folin: *Jour. Biol. Chem.*, 17, 469, 1914.
[4] Morris: *Jour. Biol. Chem.*, 21, 201 1915.

Uric Acid

1. Direct Colorimetric Method of Benedict and Franke.[1]—*Principle.*—The diluted urine is treated directly with arseno-phosphotungstic acid reagent and sodium cyanide. The blue color obtained is compared with that of a standard uric acid solution treated in the same way.

Procedure.—The urine is so diluted that 10 c.c. will contain between 0.15 and 0.30 mg. of uric acid. (Usually a dilution of 1 to 20 is satisfactory.) Ten c.c. of the diluted urine are measured into a 50 c.c. volumetric flask, 5 c.c. of the 5 per cent sodium cyanide[2] solution are added from a burette (poisonous!) followed by 1 c.c. of the arsenophosphotungstic acid reagent. The contents of the flask are mixed by gentle shaking, and at the end of 5 minutes diluted to the 50 c.c. mark with distilled water and mixed. This blue solution is then compared in a colorimeter with a simultaneously prepared solution obtained by treating 10 c.c. of the standard uric acid solution (0.2 mg. of uric acid) in a 50 c.c. flask with 5 c.c. of the sodium cyanide solution, 1 c.c. of the reagent, and diluting to the mark at the end of 5 minutes.

Calculation.—$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.2 =$ mg. of uric acid in 10 c.c. of diluted urine.

Interpretation.—For adults on a mixed diet the average excretion of uric acid is about 0.7 gram. It arises from the purines of ingested food (exogenous uric acid) and from purines derived from the body tissues by disintegration of nuclein material (endogenous uric acid). Exogenous uric acid depending entirely upon the diet is greatly increased by the ingestion of purine-rich foods (meat, liver, sweetbreads, etc.) and reduced to a very low level on purine-free foods, *e.g.*, milk, eggs, etc. (see Chapter XXXII). Endogenous uric acid is influenced by exercise and by the diet (protein foods particularly giving rise to increases). It appears to be partly the result of gastro-intestinal secretory activity.

[1] Benedict and Franke: *Jour. Biol. Chem.*, 52, 387, 1922.

[2] Solutions Required for Uric Acid Determination.—1. Reagent.—The reagent employed is the one used in the new procedure for the direct determination of uric acid in blood (Benedict, S. R.: *Jour. Biol. Chem.*, 51, 187, 1922) and is prepared as follows: 100 grams of pure sodium tungstate (preferably Merck's, or J. T. Baker's C. P. product) are placed in a liter Pyrex flask and dissolved in about 600 c.c. of water. Fifty grams of pure arsenic acid (As_2O_5) are now added, followed by 25 c.c. of 85 per cent phosphoric acid and 20 c.c. of concentrated hydrochloric acid. The mixture is boiled for about 20 minutes, cooled, and diluted to 1 liter. The reagent appears to keep indefinitely.

2. Sodium Cyanide.—A 5 per cent solution of sodium cyanide, which should be prepared fresh once in about 2 months, is used.

3. Uric Acid.—A standard solution of uric acid, acidified with hydrochloric acid, containing 0.2 mg. of uric acid in 10 c.c. is employed. This solution may be readily prepared by dilution of the phosphate standard uric acid solution described by Benedict and Hitchcock (see Chapter on Reagents and Solutions) as follows: 50 c.c. of the phosphate standard solution (containing 10 mg. of uric acid) are measured into a 500 c.c. volumetric flask and diulted to about 400 c.c. with distilled water. Twenty-five c.c. of dilute hydrochloric acid (made by diluting 1 volume of the concentrated acid with 9 volumes of water) are added, and the solution is diluted to 500 c.c. and mixed. This dilute standard solution should be prepared fresh from the phosphate standard every 10 days to 2 weeks.

On a purine-free diet the average excretion is 0.1–0.5 gram. On a high purine diet the uric acid output may be 2 grams per day.

In gout the uric acid content of the urine is low preceding an attack and increases during the attack, this fall and rise being more or less characteristic. The excretion rises after atophan administration apparently due to increased kidney activity. In leukemia the excretion is extremely high due to nuclear destruction. The uric acid content of the urine is of importance in relation to the formation of uric acid calculi. The administration of alkali carbonates and citrates by decreasing the acidity of the urine increases its solvent power for uric acid, and decreases the liability of the formation of this type of calculus.

2. Microchemical Colorimetric Method.—*Method of Folin and Wu.*[1] *Principle.*—The principle of the method depends upon the fact, first noted by Folin and Macallum[2] and further investigated by Folin and Denis,[3] and Benedict and Hitchcock,[4] that uric acid gives, with phosphotungstic acid and alkali, a deep blue color the depth of which is proportional to the amount of uric acid present. Since certain other substances present in urine may produce a similar blue color with the phosphotungstic acid, it is sometimes desirable to separate the uric acid from them. This is accomplished by precipitation as the silver salt. The silver urate is subsequently dissolved and treated with the uric acid reagent.

Procedure.—Transfer 2 to 5 c.c. of urine and about 3 c.c. of water to a centrifuge tube. Add 3 c.c. of clear acid silver lactate (5 gm. silver lactate, 5 c.c. lactic acid and 5 c.c. 10 per cent NaOH) and centrifuge for 2 to 3 minutes. Add a drop of silver lactate solution to the supernatant fluid. If a precipitate forms too much urine has been used. Repeat using smaller amount. If no precipitate forms pour off supernatant fluid as completely as possible. To a 100 c.c. volumetric flask transfer 5 c.c. of standard uric acid solution (10 c.c. of stock solution p. 379 diluted to 100 c.c.) containing 0.5 mg. uric acid. Add to the standard from a burette 2 c.c. of 15 per cent sodium cyanide (see p. 379) and add the same amount to the precipitate in the centrifuge tube. Stir the latter to complete solution, then rinse into a 100 c.c. flask using 20 c.c. of 20 per cent sodium carbonate solution from a cylinder and add 5 c.c. of water to balance that in standard. Add 20 c.c. of sodium carbonate to standard and then with shaking 5 c.c. of the uric acid reagent[5] to each flask. Let stand for 5 minutes. Then shake for a few seconds,

[1] Folin and Wu: *Jour. Biol. Chem.*, 38, 459, 1919; Folin: Laboratory Manual of Biological Chemistry, New York, 1925.

[2] Folin and Macallum: *Jour. Biol. Chem.*, 13, 363, 1912.

[3] Folin and Denis: *Jour. Biol. Chem.*, 14, 95, 1913; *ibid.*, 13, 469, 1913.

[4] Benedict and Hitchcock: *J. Biol. Chem.*, 20, 619, 1915; Benedict: *ibid.*, 20, 629, 1915.

[5] Uric acid reagent. Transfer 100 gm. sodium tungstate to a 2 liter flask and add 750 c.c. of distilled water. Shake until dissolved. A little white precipitate of calcium may remain. Add 80 c.c. of 85 per cent phosphoric acid. Close mouth of flask with a funnel and two watch glasses one small and one large and boil gently but continuously for 2 hours. If very dark in color bleach with a few drops of bromine and boil for 10–15 minutes to remove excess. Cool and dilute to 1 liter.

make to mark with water and shake vigorously for a few seconds more. After mixing pour out (or into test tubes) about 40 c.c. from each flask. This facilitates settling of the precipitate which is a decomposition product of excess uric acid reagent. Pour off clear supernatant fluids into colorimeter cups and compare. The cyanide and the prussic acid formed from it in acid solution are very poisonous. Pour all discarded solutions directly into drain pipes.

Calculation.—Since the standard contains 0.5 mg. uric acid the unknown urine will contain in the amount taken 0.5 mg. × Reading of Standard Reading of Unknown.

3. **Krüger-Schmidt Method.**—Krüger and Schmidt have devised a method for the combined determination of uric acid and the other purine bodies of urine. This procedure is described under Purine Bases, below. A modification of this method by Hunter and Givens is also described.

Purine Bases

1. **Krüger and Schmidt's Method.**—*Principle.*—This method serves for the determination of both uric acid and the purine bases. The principle involved is the precipitation of both the uric acid and the purine bases in combination with copper oxide and the subsequent decomposition of this precipitate by means of sodium sulphide. The uric acid is then precipitated by means of hydrochloric acid and the purine bases are separated from the filtrate in the form of their copper or silver compounds. The nitrogen content of the precipitates of uric acid and purine bases is then determined by means of the Kjeldahl method (see page 711) and the corresponding values for uric acid and purine bases calculated.

Procedure.—To 400 c.c. of albumin-free urine[1] in a liter flask,[2] add 24 grams of sodium acetate, 40 c.c. of a solution of sodium bisulphite[3] and heat the mixture to boiling. Add 40–80 c.c.[4] of a 10 per cent solution of copper sulphate and maintain the temperature of the mixture at the boiling-point for at least three minutes. Filter off the flocculent precipitate, wash it with hot water until the wash water is colorless, and return the washed precipitate to the flask by puncturing the tip of the filter paper and washing the precipitate through by means of hot water. Add water until the volume in the flask is approximately 200 c.c., heat the mixture to boiling and decompose the precipitate of copper oxide by the addition of 30 c.c. of sodium sulphide solution.[5] After decomposition is complete, the mixture should be acidified with acetic

[1] If albumin is present, the urine should be heated to boiling, acidified with acetic acid and filtered.

[2] The total volume of urine for the 24 hours should be sufficiently diluted with water to make the total volume of the solution 1600–2000 c.c.

[3] A solution containing 50 grams of Kahlbaum's commercial sodium bisulphite in 100 c.c. of water.

[4] The exact amount depending upon the content of the purine bases.

[5] This is made by saturating a 1 per cent solution of sodium hydroxide with hydrogen sulphide gas and adding an equal volume of 1 per cent sodium hydroxide.

Ordinarily the addition of 30 c.c. of this solution is sufficient, but the presence of an excess of sulphide should be *proven* by adding a drop of lead acetate to a drop of the solution. Under these conditions a dark brown color will show the presence of an excess of sodium sulphide.

acid and heated to boiling until the separating sulphur collects in a mass. Filter the hot fluid by means of a filter-pump, wash with hot water, add 10 c.c. of 10 per cent hydrochloric acid and evaporate the filtrate in a porcelain dish until the total volume has been reduced to about 10 c.c. Permit this residue to stand about two hours to allow for the separation of the uric acid, leaving the purine bases in solution. Filter off the precipitate of uric acid, using a small filter paper, and wash the uric acid, with water made acid with sulphuric acid, until the total volume of the original filtrate and the wash water aggregates 75 c.c. Determine the nitrogen content of the precipitate by means of the Kjeldahl method (see page 711), and calculate the uric acid equivalent.[1]

Render the filtrate from the uric acid crystals alkaline with sodium hydroxide, add acetic acid until faintly acid and heat to 70°C. Now add 1 c.c. of a 10 per cent solution of acetic acid and 10 c.c. of a suspension of manganese dioxide[2] to oxidize the traces of uric acid which remain in the solution. Agitate the mixture for one minute, add 10 c.c. of the sodium bisulphite solution[3] and 5 c.c. of a 10 per cent solution of copper sulphate and heat the mixture to boiling for three minutes. Filter off the precipitate, wash it with hot water, and determine its nitrogen content by means of the Kjeldahl method (see page 711). Inasmuch as the composition and proportion of the purine bases present in urine is variable, no factor can be applied. The result as regards these bases must therefore be expressed in terms of nitrogen.

Benedict and Saiki[4] report cases in which the total purine nitrogen by this method was less than the uric-acid nitrogen as determined by the Folin-Shaffer method. The inaccuracy was found to lie in the Krüger and Schmidt method. To obviate this they advise the addition of 20 c.c. of glacial acetic acid for each 300 c.c. of urine employed, the acid being added before the first precipitation.

Interpretation.—The amount of purine bases excreted by a normal man is small and variable. Values from 16–60 mg. have been found. The purine base nitrogen is of course only a fraction of this. The amount excreted is influenced by the diet somewhat in the same way as is the excretion of uric acid being also increased in disorders associated with increased uric acid excretion such as leukemia. The purine bases form a higher percentage of the total purine excretion in the case of the monkey, sheep, and goat than in the case of man.

2. Hunter and Givens' Modification of Krüger-Schmidt Method.[5]—*Principle.*—The Krüger-Schmidt process is combined with the microchemical colorimetric method for uric acid (see page 736).

Procedure.—The first copper-purine precipitate as obtained in the Krüger-Schmidt procedure is suspended in about 200 c.c. of water, to which there is added about 1 c.c. of concentrated hydrochloric acid. The mixture is vigorously boiled, whereupon the whole or greater part of the precipitate goes into solution.

[1] This may be done by multiplying the nitrogen value of three and adding 3.5 mg. to the product as a correction for the uric acid remaining in solution in the 75 c.c.

[2] Made by heating a 0.5 per cent solution of potassium permanganate with a little alcohol until it is decolorized.

[3] To dissolve the excess of manganese dioxide.

[4] Benedict and Saiki: *Jour. Biol. Chem.*, 7, 27, 1909.

[5] Hunter and Givens: *Jour. Biol. Chem.*, 17, 37, 1914.

Removal of the copper is effected by treatment with hydrogen sulphide in the heat, and excess of the sulphide is completely expelled by renewed boiling. Filtration under suction, and thorough washing of flask and filter result in a filtrate which is perfectly clear and nearly colorless. This is concentrated if necessary, and made up to a convenient volume which must of course be sufficiently large to retain, when cool, the uric acid in solution. Of this an aliquot part is utilized directly for the colorimetric determination of uric acid. In the remainder the residual uric acid is destroyed and bases determined according to the regular Krüger-Schmidt procedure. This modification is recommended particularly where the amount of uric acid present is minute.

3. Welker's Modification of the Methods of Arnstein and of Salkowski.[1]—*Principle.*—The phosphates are removed by treatment with magnesia mixture. The purine bases and uric acid are then thrown down as their silver salts and the nitrogen content of this precipitate determined.

Procedure.—Four hundred c.c. of urine, free from protein, are treated with 100 c.c. of magnesia mixture and 600 c.c. of water. This is then filtered and of the clear filtrate a measured quantity (600–800 c.c.) is treated with an excess (10 c.c.) of a 3 per cent silver nitrate solution. Concentrated ammonium hydroxide is added in small quantities, with stirring, until all the chlorides have dissolved. Allow the flocculent precipitate of the silver purine compounds to settle to the bottom, then pass the supernatant liquid through the filter before disturbing the precipitate. Finally transfer the precipitate quantitatively to the paper which must be of known nitrogen content. The precipitate is washed with dilute (1 per cent) ammonium hydroxide. The paper with the precipitate is then transferred to a Kjeldahl flask and about 100 c.c. of water and a small quantity (about 0.1 gram) of magnesium oxide are added. The water is then boiled until all the ammonia has been driven off. Test the steam with litmus paper.

The material in the flask is then digested by means of the usual Kjeldahl method (see page 711). The digestion must be watched carefully at the time the sulphuric acid reaches sufficient concentration to affect the filter paper, inasmuch as the SO_2 produced causes considerable frothing. The *total* nitrogen (purine base, uric acid and filter-paper nitrogen) is now determined in the usual way (see Kjeldahl Method, page 711). This result *minus* the uric acid and filter-paper nitrogen will give the figure for the purine-base nitrogen.

Allantoin

1. Method of Wiechowski-Handovsky.[2]—*Principle.*—The urine is precipitated with phosphotungstic acid and lead acetate and in the presence of chlorides with silver acetate. The heavy metals are removed with hydrogen sulphide. The allantoin is then precipitated as a mecuric compound and the amount of mercury and hence of allantoin in the precipitate determined by titration with ammonium thiocyanate. This method, though rather tedious, is probably the most accurate method for the determination of allantoin.

Procedure.—The urine is diluted to about 1 per cent urea. As rabbit urine contains in the day's output about 2–4 grams of urea, and that of other herbivora usually forms about a 4 per cent urea solution, it is usually desirable to dilute 3–4

[1] Dittman and Welker: *New York Med. Jour.*, May–June, 1909.

[2] Handovsky: *Zeit. physiol. Chem.*, 90, 211, 1914. Christman and Lewis: *Jour. Biol. Chem.*, 57, 379, 1923.
Wiechowski: Neubauer-Huppert: Analyse des Harns, Wiesbaden, 1913, p. 1076.

times. A greater dilution is not desirable. The urine is treated with 1 per cent of sulphuric acid and about 3 c.c. of acetic acid for each day's volume. Test a small quantity of the urine to determine the amount of 50 per cent phosphotungstic acid required to completely precipitate it. The bulk of the urine is then treated on this basis with sufficient solid phosphotungstic acid to precipitate it completely. Stir to dissolve the acid and allow to stand for several hours. Filter with suction, first lining the filter with infusorial earth by rubbing up a little of the substance with some of the urine mixture and filtering with suction. To some ordinary lead oxide in a mortar add a small amount of the filtrate and stir until it becomes warm, then add the rest of the filtrate and stir, adding more oxide if necessary until the solution reacts alkaline due to the formation of basic lead acetate.

Filter again. The filtrate should give no precipitate with basic lead acetate. A measured volume of the filtrate is then treated with measured volumes of acetic acid and silver nitrate solution to completely precipitate any chlorides present. Filter again preferably through infusorial earth. Pass hydrogen sulphide through the filtrate until the heavy metals are completely precipitated. Filter again. Pass a current of air through the filtrate to remove all hydrogen sulphide (test with lead acetate paper). Neutralize this final filtrate with calcium carbonate and remove the carbon dioxide with a current of air.

The neutral filtrate is then treated with an amount of allantoin reagent in excess of that required to precipitate the allantoin as indicated by a preliminary test. (The allantoin reagent is a solution containing 5 per cent mercuric acetate and 20 per cent of sodium acetate. The reagent when used must be clear.)

Allow to stand for half an hour (not too long) and then filter. A measured amount of the filtrate is taken and treated with about 10 c.c. of iron-ammonium alum and the red solution decolorized with dilute sulphuric acid. (The solution is not completely decolorized but retains a faint greenish tint.) Any calcium sulphate precipitate formed at this point may be disregarded. Titrate with N/10 ammonium thiocyanate solution to a yellow color, which increases in intensity on the addition of 1–2 drops more of solution. The titration should not be carried out in artificial light and some practice is required to get the exact end point. The thiocyanate solution should be standardized occasionally against standard silver nitrate solution.

Calculation.—One c.c. of N/10 NH_4CNS solution corresponds to 0.00436 gram of allantoin. The limit of error of the method is about 5 mg. for the daily output of allantoin. In the calculation of course all losses through filtration, etc., must be considered.

For the considerable modifications required in carrying out the determination on human urine with its very low content of allantoin see the section by Wiechowski in Neubauer-Huppert.[1]

Interpretation.—Allantoin may be found in very small amounts in human urine (5–15 mg. per day), appearing to be mainly, though not entirely, exogenous in origin. It forms, however, the principal end-product of the purine metabolism of practically all mammals other than man and the anthropoid apes. Thus over 90 per cent of the purine-allantoin nitrogen excretion of the dog, the cow, and the pig occurs in this form. In these animals its origin is from exogenous and endogenous purines, and its excretion is influenced by much the same factors as is that of uric acid in man.

[1] Wiechowski: Neubauer-Huppert: Analyse des Harns, Wiesbaden, 1913, p. 1076.

2. Determination by Difference.—Method of Plimmer and Skelton.[1]—Allantoin is transformed into urea and determined as such by the acid-magnesium chloride method of Folin for urea in urine.[2] Urease, however, has no effect upon allantoin. Therefore, determine the urea + allantoin of the urine according to Folin's procedure, and also determine the true urea according to the urease method (see Urea). The difference between the results thus obtained represents allantoin. If sugar is present it must be removed before applying Folin's procedure.

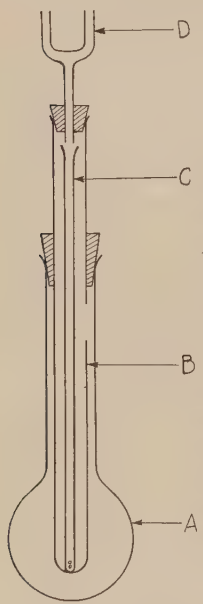


FIG. 229.—CONTINUOUS EXTRACTION APPARATUS (*Griffith*).

(A), 500 c.c. Kjeldahl flask. (B), glass tube (420 × 15 mm.) with side opening. (C), glass tube (400 × 6 mm.) with widened top and small openings at bottom. (D), condenser.

Hippuric Acid

1. Method of Griffith.[3]—Principle.—Hippuric acid is extracted from urine with ether in a continuous extraction apparatus. The residue obtained by distilling off the ether is treated with bromine and sodium hypobromite to destroy traces of urea. Hippuric acid nitrogen is then determined in the residue by the Kjeldahl procedure.

Procedure.—Place 10 c.c. of protein-free urine (containing not more than 150 mg. of hippuric acid) and 0.1 c.c. of conc. HCl in the extraction tube and 100 c.c. of ether in the 500 c.c. Kjeldahl flask. (The arrangement of the extraction apparatus is shown in Fig. 229.) Immerse the lower portion of the flask in a water bath heated to 60–70°C. and continue the extraction for one hour. Distil off the ether. To the dry residue in the flask add 5 c.c. of sodium hypobromite[4] solution and shake one minute. Add 2 c.c. of sulphuric acid (diluted 1:5). Mix thoroughly. Add 2 c.c. of 25 per cent NaOH and 2 c.c. of the hypobromite solution and shake one minute. Proceed with the determination of nitrogen in the flask by the Kjeldahl method.

Calculation.—If (y) represents the number of c.c. of 0.1 N acid neutralized by ammonia, then

$$y \times 1.4 \times \frac{179}{14} = \text{mg. of hippuric acid in 10 c.c. of urine.}$$

A method also involving ether extraction has been suggested by Quick.[5] The older method of Folin and Farmer (*Jour. Biol. Chem.*, 11, 257, 1912) involves the hydrolysis of the hippuric acid and the titration of benzoic acid by sodium ethylate. This method was later improved by Kingsbury and Swanson (*Jour. Biol. Chem*, 48, 13, 1921).

[1] Plimmer and Skelton: *Bioch. J.*, 8, 70 and 641, 1914.

[2] Procedure.—To 5 c.c. of urine in a 200 c.c. flask add 5 c.c. conc. HCl, 20 gm. $MgCl_2$ crystals, and a small cube of paraffin. Attach a reflux condenser. Leave water jacket empty. Heat to boiling until marked frothing occurs. Fit a thistle tube to the top of the condenser. Then boil gently for 2 hours with water running through condenser. Rinse condenser with 50 c.c. of water. Make slightly alkaline with NaOH and distill off ammonia as in the Kjeldahl method. Anhydrous allantoin contains 35.44 per cent nitrogen.

For discussion see Harding and Young: *Jour. Biol. Chem.*, 40, 231, 1919 and Morgan and Osburn: *Jour. Biol. Chem.*, 66, 577, 1925.

[3] Griffith: *Jour. Biol. Chem.*, 69, 197, 1926. Also private communication.

[4] Prepared by mixing equal volumes of 25 per cent NaOH and a solution containing 12.5 gm. of bromine and 12.5 gm. of sodium bromide dissolved in 100 c.c. of water.

[5] Quick: *Jour. Biol. Chem.*, 67, 477, 1926.

Interpretation.—The average excretion of hippuric acid by a normal adult man is about 0.7 gram per day. The amount is increased by the ingestion of benzoic acid or fruits containing it (plums, prunes, cranberries). It arises in part apparently from putrefaction products formed in the intestine. In herbivora it is often the most abundant nitrogenous constituent of the urine.

Lactic Acid

1. **Method of Clausen.**[1] *Principle.*—Lactic acid is extracted from the urine with ether. By treatment with sulphuric acid acetaldehyde is formed. $CH_3CHOHCOOH \rightarrow CH_3CHO + CO + H_2O$. The aldehyde is combined with sodium bisulphite. $CH_3CHO + NaHSO_3 \rightarrow CH_3CHOHSO_3Na$. The bound sulphite is titrated with standard iodine solution.

Procedure.—If the urine contains albumin add to 5 c.c. of it 0.5 c.c. of 10 per cent sodium tungstate and 0.5 c.c. N sulphuric acid. Place 2 c.c. of filtrate or albumin free urine in an extraction tube of the type shown in Fig. 229. Use a 300 c.c. Kjeldahl flask. The tube B should be narrowed at the lower end so that 2 c.c. forms a column about 10 cm. deep. Introduce 50 c.c. of ether into the flask. Add to the urine a few drops of strong phosphate buffer solution of pH 7.0. Extract for 15 minutes to remove free phenols. Then add 1.3 gm. ammonium sulphate and a few drops of concentrated sulphuric acid. Extract with fresh ether for 30 minutes. To the ether extract add about 10 c.c. of water 1 drop of 5 per cent phenolphthalein and enough 0.1 N NaOH to just give an alkaline reaction. Distill off the ether. Transfer contents of flask to a small beaker and evaporate on a water bath nearly but not quite to dryness. Wash with a small quantity of water into a Pyrex tube (20 × 200 mm.). Arrange for aspiration. A straight inlet tube drawn to a capillary extends nearly to the bottom. The outlet tube is joined directly to a 40 cm. air condenser (a glass tube) which in turn leads to two receiving tubes (20 × 200) entirely similar to the distillation tube mentioned. The receiving tubes each contain 20 c.c. of 0.02 N $NaHSO_3$.[2] Rubber stoppers are used. Four sets of determinations are usually run simultaneously. The capillary inlet tubes should have nearly the same diameters. Air pressure or a suction pump may be used to give a rapid but uniform current of air. Add 10 c.c. of 1:1 sulphuric acid (by volume) slowly with constant cooling. Distill for about one hour by putting reaction tube in a fusible alloy bath kept at 150°. It is best to have a thermometer with bulb immersed in the reaction mixture so that the temperature may be kept at 145–150°. Water is added to keep the acid concentration at about 1:1 throughout the distillation. Transfer contents of receiver to a flask and oxidize the excess bisulphite by adding 0.1 N iodine to the first definite blue with the small amount of soluble starch solution used as an indicator. Add enough saturated sodium bicarbonate solution to discharge the

[1] Clausen: *Jour. Biol. Chem.*, 52, 263, 1922.

[2] *Sodium acid sulphite* is prepared by leading SO_2 gas into a saturated Na_2SO_3 solution as long as CO_2 is given off. Dilution is made only as needed. *Iodine* solutions are made by diluting 0.1 N iodine with 2 per cent KI solution. For preparation and standardization see "Reagents and Solutions." *Pure zinc lactate* for checking the procedure is made by adding to a solution of lactic acid previously neutralized with NaOH the calculated amount of zinc chloride in solution to form $Zn(C_3H_5O_3)_2\ 3H_2O$. Recrystallize four times. A 0.1 N solution contains 14.88 gm. per liter.

blue color. Titrate the bound bisulphite with 0.01 to 0.001 N iodine to an end point determined by a blank control run in the same manner as above but without urine. Subtract the number of c.c. required for the blank.

Calculation.—One c.c. 0.01 N iodine = 0.45 mg. lactic acid as 2 atoms of iodine are required per molecule of acid.

Interpretation.—Normally the urine contains 5–13 mg. per 100 c.c. These values are increased after severe exercise and probably in such pathological conditions as are accompanied by deficient oxidative metabolism. For discussion of lactic acid in urine see p. 563.

Conjugated Glycuronic Acids

Methods of Quick[1] and Csonka.[2]—A satisfactory quantitative method which is applicable to the analysis of all conjugated glycuronic acids is not available. Quick has described a method for menthol glycuronic acid in urine which, he claims, can be extended, with slight modifications, to the determination of other conjugated glycuronic acids. It consists in extraction of the compound with ether, hydrolysis with dilute acid and finally determination of the liberated glycuronic acid by a copper reduction method. A method for benzoyl glycuronic acid, similar to the above, has been reported by Csonka.

Glucose[3]

1. Benedict's Method.[4]—*Principle.*—Benedict's reagent for the estimation of reducing sugars contains potassium thiocyanate as well as copper sulphate, and in the presence of the former a white precipitate of cuprous thiocyanate is formed on reduction instead of the usual red precipitate of cuprous oxide. The small amount of potassium ferrocyanide also aids in keeping cuprous oxide in solution. As the precipitate formed is white the loss of all blue tint in the solution, indicating complete reduction of the copper, is readily observed. The alkali used is sodium carbonate, which has the advantage over the hydroxides in that there is less danger of destruction of small amounts of sugar. The solution also has the great advantage of being stable for an indefinite length of time. The method is recommended for simplicity and accuracy.

Procedure.—The urine, 10 c.c. of which should be diluted with water to 100 c.c. (unless the sugar content is believed to be low, when it may be used undiluted), is poured into a 50 c.c. burette up to the zero mark. Twenty-five c.c. of

[1] Quick: *Jour. Biol. Chem.*, 61, 667, 1924.

[2] Csonka: *Jour. Biol. Chem.*, 60, 545, 1924.

[3] The method for sugar adopted by the Committee on Urinary Impairments of the Association of Life Insurance Medical Directors of America may be found on page 751.

[4] Benedict: *Jour. Am. Med. Ass'n*, 57, 1193, 1911. Benedict's colorimetric method for sugar in blood (see pp. 383 and 754) may be applied to urine after treatment with Lloyd's earth (p. 753).

the reagent[1] are measured with a pipette into a porcelain evaporation dish (25–30 cm. in diameter), 10 to 20 grams of crystallized sodium carbonate (or one-half the weight of the anhydrous salt) are added, together with a small quantity of powdered pumice stone or talcum, and the mixture heated to boiling over a free flame until the carbonate has entirely dissolved. The diluted urine is now run in from the burette, rather rapidly, until a chalk-white precipitate forms and the blue color of the mixture begins to lessen perceptibly, after which the solution from the burette must be run in a few drops at a time, until the disappearance of the last trace of blue color, which marks the end point. The solution must be kept vigorously boiling throughout the entire titration. If the mixture becomes too concentrated during the process, water may be added from time to time to replace the volume lost by evaporation.

Calculation.—The calculation of the percentage of sugar in the original sample of urine is very simple. The 25 c.c. of copper solution are reduced by exactly 50 mg. of glucose. Therefore the volume run out of the burette to effect the reduction contained 50 mg. of the sugar. When the urine is diluted 1:10, as in the usual titration of diabetic urines, the formula for calculating the per cent of the sugar is the following:

$$\frac{0.050}{X} \times 1000 = \text{per cent in original sample, wherein X}$$

is the number of cubic centimeters of the diluted urine required to reduce 25 c.c. of the copper solution.

In the use of this method chloroform must not be present during the titration. If used as a preservative in the urine it may be removed by boiling a sample for a few minutes, and then diluting to its original volume.

Interpretation.—Sugar in the urine in amounts sufficient to be detected by the commonly employed qualitative tests indicates a pathological condition, unless very large amounts of sugar have been ingested just previously, in which case the condition is spoken of as an alimentary glycosuria. Persistent glycosuria thus indicates diabetes mellitus, a disorder in which the amount of sugar may rise as high as 10 per cent and averages 3–5 per cent. The volume of urine excreted per day is usually also large and the absolute sugar excretion may thus be very great (100 grams of glucose per day are not uncommon). The quantitative methods for the estimation of sugar in urine enable us to determine the severity of the disorder as well as to follow its course under treatment, etc.

[1] Copper sulphate (crystallized) ..... 18.0 grams.
Sodium carbonate (crystallized, one-half the weight of the anhydrous salt may be used) ..... 200.0 grams.
Sodium or potassium citrate ..... 200.0 grams.
Potassium thiocyanate ..... 125.0 grams.
Potassium ferrocyanide (5 per cent solution) ..... 5.0 c.c.
Distilled water to make a total volume of ..... 1000.0 c.c.

With the aid of heat dissolve the carbonate, citrate and thiocyanate in enough water to make about 800 c.c. of the mixture and filter if necessary. Dissolve the copper sulphate separately in about 100 c.c. of water and pour the solution slowly into the other liquid, with constant stirring. Add the ferrocyanide solution, cool and dilute to exactly 1 liter. Of the various constituents, the copper salt only need be weighed with exactness. Twenty-five c.c. of the reagent are reduced by 50 mg. of glucose.

2. Sumner's Method.[1] *Principle.*—Urine is heated with a dinitrosalicylic acid reagent which is reduced by the sugar, and the resultant color compared with standards. This method is applicable to normal as well as glycosuric urine.

Procedure.—Pipette into a Folin-Wu blood sugar tube 1 c.c.of urine (diluted if necessary) and 3 c.c. of the dinitrosalicylic acid reagent.[2] Mix and heat 5 minutes in boiling water. Cool 3 minutes in running water, dilute to 25 c.c., mix and compare in a colorimeter with a standard glucose solution treated simultaneously in the same way. 0.1 per cent glucose is a satisfactory concentration for the standard solution. If the color obtained with the urine is too dark, repeat the test using more dilute urine.

Calculation:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \frac{0.1}{100} \times \frac{100}{1} \times \text{dilution} = \text{per cent glucose}$$

Or

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \frac{\text{Dilution}}{10} = \text{per cent glucose}$$

3. Folin-McEllroy-Peck Method.[3] *Principle.*—The method is a titration procedure depending upon the use of an alkaline copper solution in which the cupric hydroxide is held in solution by means of phosphate instead of the customary tartrates, citrates, or glycerol. The method is applicable to the determination of lactose in milk.

Procedure.—Place 5 c.c. of an acidified 5.9 per cent copper sulphate solution[4] in a large, hard glass test tube and add approximately 1 c.c. of 20 per cent sodium carbonate solution. Shake for a moment and add 4 to 5 gms. of phosphate-carbonate-thiocyanate mixture[5] and a small pebble. Heat gently with shaking until all the salts have dissolved except for a few isolated particles of sodium carbonate. A clear solution is usually obtained in less than 1 minute at temperatures which need not exceed 60°C. From a burette[6] add undiluted urine

[1] Sumner: *Jour. Biol. Chem.*, 65, 383, 1925.

[2] To 10 gm. of crystallized phenol add 22 c.c. of 10 per cent NaOH. Dissolve in a little water and dilute to 100 c.c. Weigh 6.9 gm. of sodium bisulphite and add to this 69 c.c.] of the alkaline phenol solution. To this add a solution containing 300 c.c. of 4.5 per cent NaOH, 255 gm. of Rochelle salt ($NaKC_4H_4O_6 \cdot 4H_2O$) and 880 c.c. of 1 per cent dinitrosalicylic acid. Mix and keep tightly stoppered in well filled bottles. Dinitrosalicylic acid may be obtained from the Eastman Kodak Co., Rochester, N. Y., or may be prepared from the directions given by Sumner, *Jour. Biol. Chem.*, 47, 5, 1921.

[3] Folin and McEllroy: *Jour. Biol. Chem.*, 33 513, 1918; Folin and Peck: *Jour. Biol. Chem.*, 38, 287, 1919.

[4] Prepare this acidified copper sulphate solution by dissolving 59 gm. of $CuSO_4 \cdot 12H_2O$ in water together with 2 c.c. of concentrated sulphuric acid and making up to 1 liter. Five c.c. of this solution correspond to 25 mg. of glucose or fructose, 45 mg. of anhydrous maltose, or 40.4 mg. of anhydrous lactose.

[5] To prepare the phosphate-carbonate-thiocyanate mixture powder in a large mortar 200 gm. of crystallized disodium phosphate ($Na_2HPO_4 \cdot 12H_2O$) and sprinkle over it about 50 gm. of sodium thiocyanate (or 60 gm. of potassium thiocyanate). Mix for 10 minutes with pestle and spoon, giving a uniform semi-liquid paste. Add about 120 gm. of monohydrated sodium carbonate (or 100 to 110 gm. of anhydrous carbonate) and mix with pestle and spoon until a rather fluffy, granular powder is obtained. To test the completeness of the mixing add 5 gm. of the powder to 5 c.c. of the copper solution; if any black specks are formed, even temporarily, the mixing is incomplete. A certain amount of green color is, however, practically unavoidable when this test is applied. If no black coloration is obtained allow the mixture to stand in the mortar for a few hours or over night (covered with paper) mix once more and transfer to bottles. In stoppered bottles the mixture keeps indefinitely.

[6] Special 5 c.c. sugar burettes graduated in 0.02 c.c. provided with accessory capillary tips for delivering very small drops are accessible on the market.

(0.4 c.c. to 1.0 c.c.) heat fairly rapidly to boiling and boil very gently for 2 minutes. With the full required amount of sugar present at the beginning (the 5 c.c. of copper solution are reduced by 25 mg. of glucose), the boiling solution becomes suddenly turbid within 5 seconds after the boiling point has been reached. If, within the first 15 seconds of boiling, the contents of the test tube do not thus suddenly become filled with the cuprous thiocyanate precipitate, then less than half the required amount of sugar has been added and more urine should be added without further delay and the gentle boiling be continued. On the other hand, when an excess of sugar has been inadvertently added at the beginning of the process it is advisable to note the time required for complete decolorization of the copper, for this time (see table) can serve as a guide to the quantity of solution to be introduced at the next titration.

The only restriction called for in the final titration is that complete reduction must not occur in less than 4 minutes of boiling. It makes practically no difference in the result (at the most 1 per cent) if the boiling period be prolonged to 8 or 9 minutes, provided that the boiling be gentle enough to prevent excessive concentration. The volume of the solution in the test tube should not become less than 6 to 7 c.c.

Calculation.—Divide 2.5 by the volume of urine taken (whether this is several c.c. or a fraction of 1 c.c.) to get the per cent of sugar in the urine.

TIME OF BOILING REQUIRED FOR COMPLETE REDUCTION OF COPPER SOLUTION BY AN EXCESS OF GLUCOSE

Glucose mg.	Boiling time minute	seconds
50	0	25
40	0	40
35	0	55
30	1	20 to 30
27.5	1	30 to 55

Interpretation.—See page 745.

4. **Peters' Method.**[1]—*Principle.*—The sugar solution is boiled with an alkaline copper solution under rigidly standardized conditions and after filtration the unreduced copper is determined by adding potassium iodide and titrating the liberated iodine with standard thiosulphate solution.

Procedure.—A. The Heating Power.—It is necessary to standardize the heating power of the flame used in the reduction process. A 200 c.c. Erlenmeyer flask of Pyrex glass and of about 6 cm. basal diameter is used. This bears a two-hole rubber stopper, one hole of which carries a thermometer. The lower end of the thermometer should extend to about 2 mm. from the bottom of the flask and the 35° mark on the thermometer stem should be visible above the stopper. The flask is placed on an asbestos gauze supported by an adjustable ring stand. A Bunsen or Méker burner is used and is placed at from 3–5 cm. beneath the lower surface of the asbestos gauze. Use a large flame and allow the ring and gauze to become thoroughly

[1] Peters: *J. Am. Chem. Soc.*, 34, 928, 1912; 34, 422, 1912.

heated. Then place the flask, into which 60 c.c. of distilled water has been introduced, in the center of the gauze and observe the time required for the temperature in the flask to rise from 35 to 95°C. By several trials the flame and position of the gauze are so adjusted that it requires (within a few seconds either way) just 120 seconds for the temperature to rise from 35–95°C. This standard flame is then used in the determinations which follow.

B. The Reduction Process.—Into a 200 c.c. Jena Erlenmeyer flask fitted as above, introduce 20 c.c. of alkaline tartrate solution,[1] 20 c.c. of the copper sulphate solution[1] and the sugar solution to be analyzed which should first be made up to 20 c.c. so that the total volume of the fluid in the reduction flask is always 60 c.c. Place the flask over the standard flame and note when the temperature of the mixture reaches 95°C. Allow to boil for exactly 20 seconds after the temperature reaches 95°. Then the flask is promptly removed with the stopper still in place and held under the tap for a moment to stop the reduction but not to cool the mixture more than a few degrees below the boiling-point. Filter hot through a previously prepared Gooch crucible with a heavy asbestos mat through which sufficient of a suspension of talcum has been filtered so that with suction, not a stream but a rapid succession of drops comes through the filter. (A calcium chloride tube packed with glass wool and asbestos may also be used.) The suction flask should have a capacity of about 200 c.c. so that the titration may be carried out in it directly. Wash the filter with a fine stream of water using not more than 15–20 c.c. of water in all. To the filtrate add 4 c.c. of concentrated sulphuric acid and cool to 20°C. Add 6–7 c.c. of a saturated solution of potassium iodide. Titrate the liberated iodine with standard thiosulphate solution,[2] adding a few drops of a solution of soluble starch as an indicator near the close of the titration. The "spot test" may be used in determining the end point. As long as a drop of thiosulphate produces a perceptible white area in falling upon the quiet solution the end point has not been reached. The chocolate-brown color of the mixture changes to a light cream white as the last necessary drop of thiosulphate is added.

A blank should be run in exactly the same way but with the omission of the sugar solution.

Calculation.—Subtract the number of cubic centimeters of thiosulphate required for the titration of unreduced copper from the number of cubic centimeters required for the blank. This gives the amount of thiosulphate equivalent to copper reduced. Multiply this result by the value of 1 c.c. of thiosulphate expressed as Cu, and obtain the number of milligrams of copper reduced. Then by consulting the table of values (below) determine the weight of glucose equivalent to this amount of copper.

[1] *Copper Solution.*—Dissolve 34.639 grams of highest purity crystallized copper sulphate (Kahlbaum's "zur Analyse mit Garantieschein) in water to make 500 c.c.

Alkaline Tartrate Solution.—Dissolve 173 grams of sodium potassium tartrate and 125 grams of potassium hydroxide in water to make 500 c.c.

[2] N/5 *Sodium Thiosulphate.*—Dissolve about 50 grams of ordinary c.p. sodium thiosulphate or exactly 49.66 grams of the pure, dry, recrystallized salt, in enough boiled-out distilled water to make a liter. Allow to stand for several days. The solution should be standardized against the copper solution prepared as above. For this purpose introduce 20 c.c. of the copper solution into a 200 c.c. Erlenmeyer flask, add 20 c.c. of 30 per cent acetic acid and 40 c.c. of water. Add about 7 grams of a saturated solution of KI and titrate with the thiosulphate using starch as an indicator. Calculate the equivalent of 1 c.c. of thiosulphate in Cu. One c.c. of the copper sulphate solution contains 17.647 mg. of Cu. The thiosulphate remains constant for some months. It should be kept in a dark bottle.

Cole[1] has determined the copper values for lactose using this method exactly as outlined. He has also suggested the following empirical formula which agrees well with the values derived from his table:

$$\text{Mg. anhydrous lactose} = 1.25 + 0.778 \times \text{mg. Cu.}$$

Interpretation.—See page 745.

TABLE FOR THE DETERMINATION OF GLUCOSE

According to Peters

Glucose	Copper, mg.	Glucose / Cu ratio	Glucose	Copper, mg.	Glucose / Cu ratio
1	1.2	0.833	60	115.5	0.522
2	2.8	0.714	70	134.4	0.522
5	8.2	0.610	80	152.9	0.522
8	13.8	0.580	90	171.0	0.522
10	17.4	0.575	100	191.6	0.522
15	27.7	0.542	110	208.9	0.527
20	37.1	0.539	120	228.1	0.526
25	48.1	0.522	135	255.0	0.529
30	57.3	0.522	150	280.8	0.534
35	67.6	0.522	165	306.8	0.538
40	76.2	0.522	180	330.5	0.545
45	86.0	0.522	200	349.6	0.572
50	96.0	0.522			

5. Power and Wilder's Method.—*Principle.*—Partial reduction of an excess of alkaline ferricyanide to ferrocyanide is employed, and the unreduced ferricyanide is determined by thiosulphate titration of the I_2 which is quantitatively liberated on the addition of potassium iodide-zinc sulphate-acetic acid, as in the Hagedorn-Jensen blood method (see p. 385). This procedure is applicable with exactness only to the practically nitrogen-free filtrate from urine which has been precipitated by a mercury salt. By such clearing identical reducing values, calculated as glucose, have been obtained by both the ferricyanide method and Shaffer-Hartmann's residual titration,[2] with which the former compares in convenience and accuracy. The advantageous features of the ferricyanide titration are, a very sharp, easily approached end point, and the fact that there is no danger of reoxidation of the reduction product, potassium ferrocyanide. Due to its sensitivity to other substances, it cannot claim the general usefulness of the copper method.

Procedure.—To one volume of urine in a 150 c.c. beaker or Erlenmeyer, add two volumes of mercuric sulphate,[3] a few drops of toluene to prevent excessive foaming, and neutralize carefully with solid sodium bicarbonate until alkaline to litmus. Filter, and remove traces of mercury from the filtrate by adding mag-

[1] Cole: *Biochem. J.*, 8, 134, 1914.

[2] Shaffer and Hartmann: *Jour. Biol. Chem.*, 45, 349, 1925.

[3] An almost ½ molar mercuric sulphate solution may be prepared by dissolving ½ mole HgO in 1000 to 1200 c.c. of 5 N sulphuric acid. About 1 equivalent of the acid may be neutralized with strong NaOH before a permanent precipitate forms. Mercuric nitrate is not suitable for clearing because of the subsequent reduction of nitrate ion to nitrite. Mercuric acetate is not used because a high concentration of acetate ion exerts a reducing effect on ferricyanide.

nesium powder (heaped up on the end of a small spatula blade), and a drop or two of concentrated HCl. Allow to stand 4 to 5 minutes and filter through paper with pores sufficiently small to retain the finely divided mercury precipitate. The filtrate, which may contain from 1 or 2 to 50 mg. of glucose, is measured into a 200 c.c. pyrex Erlenmeyer flask, water is added to make a volume of 90 c.c., then 10 c.c. of alkaline ferricyanide.[1] Heat exactly 10 minutes in a water bath of such size that boiling does not stop on introduction of the cold flask. Cool immediately in running water, add 10 c.c. of potassium iodide solution[2] then 10 c.c. of acid zinc sulphate solution,[3] and titrate the liberated iodine with standard thiosulphate,[4] adding indicator[5] only when the iodine color has faded to light yellow. Determine the blank value of the ferricyanide similarly (heating not necessary).

Calculation.—Express each thiosulphate titration in terms of c.c. of 0.1 N solutions, and subtract the volume required in the unknown from that required by the blank. This difference represents the c.c. of 0.1 N ferricyanide reduced by the sugar in unknown, and the corresponding milligrams of glucose may be obtained from a curve constructed from the results of several determinations with known amounts of glucose, or from the following table. Several determinations may be conducted simultaneously. The blank value of the ferricyanide solution changes very slowly, and should be checked every 2 or 3 days.

RELATION BETWEEN GLUCOSE AND FERRICYANIDE

Mg. glucose	C.c. 0.1 N ferricyanide reduced
1.00	0.37
3.00	0.95
5.00	1.56
10.00	3.06
20.00	6.12
30.00	9.20
40.00	12.30
50.00	15.37

On diabetic urines, uncleared, ferricyanide-glucose values are consistently 0.20 to 0.30% high, absolute; i.e., a urine containing 4.00% glucose will on analysis give 4.25%. Diabetic urine sugars may thus be conveniently routinely

[1] Approximately 0.2 N potassium ferricyanide in 0.2 N potassium carbonate (chemicals of C.P. grade used without further purification). This solution should be made in artificial light, both the carbonate and ferricyanide filtered (the latter never filtered hot), made up to volume, and placed in a brown glass bottle, and thereafter carefully shielded from light. A siphon protected from light throughout its length by rubber tubing, and connected with a ten c.c. automatic pipette, affords a convenient arrangement for rapid measurement without undue exposure. It is remarkable that a short exposure to light of only 5 to 10 minutes reduces the oxidizing power of ferricyanide for glucose, but apparently does not affect its ability to liberate iodine from potassium iodide.

[2] 30% potassium iodide. This solution rarely develops free iodine if kept in dark glass; part of a drop of 0.1 N thiosulphate is usually sufficient to react with the small amounts that sometimes appear.

[3] Zinc sulphate-acetic acid containing 150 g. $ZnSO_4 \cdot 7H_2O$, and 150 c.c. glacial acetic acid per liter, and 50 to 100 g. NaCl.

[4] An accurately standardized thiosulphate solution, about 0.1 N.

[5] Indicator: 0.1 to 1% starch solution containing sodium chloride.

determined to within an accuracy of 1 to 5% by subtracting an absolute correction of 0.25 from the percentage obtained by direct application of the ferricyanide method. More than 5 or 10 c.c. of to 10 dilution should not be used, otherwise urea becomes a disturbing factor. Urea alone does not reduce ferricyanide under the conditions of the method.

6. Benedict's Picrate Method. (Adopted by the Committee on Urinary Impairments of the Association of Life Insurance Medical Directors of America.) Principle.—The color produced by the reduction of picric acid (p. 78) is compared with permanent inorganic standards representing definite concentrations of sugar.

Procedure.—Measure 1 c.c. of urine into a test tube graduated at 25 c.c. Add 3 c.c. of picric acid solution (2 grams of pure dry picric acid per liter) and 0.5 c.c. of 5 per cent NaOH. Add next 5 drops of 50 per cent acetone solution (prepared fresh each day by diluting acetone with an equal volume of water) and place the tube promptly in a boiling water bath. In 12 minutes remove the tube, cool and dilute the contents to 25 c.c. Compare this colored solution with the permanent standards in tubes of the same dimensions and estimate the amount of sugar. The permanent sugar standards are made as follows:

Permanent Sugar Standards.[1]

Sugar	Ferric chloride solution	Cobalt chloride solution	Dilute hydrochloric acid	Water
0.1%	18 c.c.	7 c.c.	8 c.c.	To make 100 c.c.
0.2%	28 c.c.	13 c.c.	8 c.c.	
0.3%	22 c.c.	22 c.c.	8 c.c.	
0.4%	16 c.c.	30 c.c.	8 c.c.	
0.5%	14 c.c.	40 c.c.	8 c.c.	

Put each standard in test tubes of same diameter as used by sugar determination.

7. Fermentation Method.—*Principle.*—This method consists in the measurement of the volume of carbon dioxide evolved when the dextrose of the urine undergoes fermentation with yeast.[2] None of the various methods whose manipulation is based upon this principle

[1] Solutions needed.

(1) *Ferric Chloride* (Merck's Analyzed)

Dissolve 200 gm. ferric chloride [$FeCl_3\ 6H_2O$] in about 300 c.c. distilled water, transfer to a 500 c.c. volumetric flask, make up to volume with distilled water and mix well. Filter through a dry filter paper.

(2) *Cobalt Chloride* (analyzed grade).

Dissolve 150 gr. cobalt chloride [$CoCl_2 \cdot 6H_2O$] in about 300 c.c. distilled water, transfer to a 500 c.c. volumetric flask, make up to volume with distilled water and mix well. Filter through a dry filter paper.

(3) *Dilute Hydrochloric Acid.*

Dilute 5 c.c. concentrated hydrochloric acid (about 37%) to 50 c.c. with distilled water.

[2] See Castellani and Tayler's suggestion, p. 643.

is *absolutely* accurate. The method in which Einhorn's saccharometer (Fig. 19, page 79) is the apparatus employed is perhaps as satisfactory as any for clinical purposes.

Procedure.—Place about 15 c.c. of urine in a mortar, add about 1 gram of yeast (1/16 of the ordinary cake of compressed yeast) and carefully crush the latter by means of a pestle. Transfer the mixture to the saccharometer, being careful to note that the graduated tube is completely filled and that no air bubbles gather at the top. Allow the apparatus to stand in a warm place (30°C.) for 12 hours and observe the percentage of dextrose as indicated by the graduated scale of the instrument. Both the percentage of dextrose and the number of cubic centimeters of carbon dioxide are indicated by the graduations on the side of the saccharometer tube.

The fermentation method becomes a much more accurate procedure if the saccharometer of Lohnstein is used.[1]

The availability of the fermentation procedure as a quantitative aid has been appreciably lowered through the important findings of Neuberg and associates.[2] They show that yeast has the property of *splitting off carbon dioxide from the carboxyl group of amino- and other aliphatic acids*. The active agent in this "sugar-free fermentation" is an enzyme called *carboxylase*. Inasmuch as amino-acids are always present in the urine, the error from this source is apparent.

7. Polariscopic Examination.—Before subjecting urine to a polariscopic examination the slightly acid fluid should be decolorized as thoroughly as possible by the addition of a little basic lead acetate. The urine should be well stirred and then filtered through a filter paper which has not been previously moistened. In this way a perfectly clear and almost colorless liquid is obtained.

In determining dextrose by means of the polariscope it should be borne in mind that this carbohydrate is often accompanied by other optically active substances, such as proteins, fructose, β-hydroxybutyric acid, and conjugate glycuronates which may introduce an error into the polariscopic reading; the method is, however, sufficiently accurate for practical purposes.

For directions as to the manipulation of the polariscope see page 80.

Below are given the specific rotations of some physiologically important sugars as well as of certain other optically active substances the possible presence of which must be borne in mind in determining glucose polarimetrically in urine.

	Specific Rotation		Specific Rotation
Glucose	+52.49	Fructose	−92.25
Maltose	+136.5	β-Hydroxybutyric acid.	−24.12
Isomaltose	+68.0		
Lactose	+52.53	Conjugated Glycuronic Acids.	Levorotatory in varying degrees.
Pentose (i-arabinose).	0.0		

[1] Lohnstein: *Münch. med. Woch.*, 1899, 1671.
[2] Neuberg and associates: *Biochem. Zeitschr.*, vol. 31 and 36, 1911.

DETERMINATION OF SUGAR IN NORMAL URINE[1]

1. Benedict and Osterberg's Method for Sugar in Normal Urine.[2]—*Principle.*—The red color obtained by heating a glucose solution with picric acid and sodium carbonate is employed as the basis of the colorimetric determination. Acetone is used to eliminate color due to creatinine.

Procedure.—To 15 c.c. of urine (diluted to a sp. gr. of not over 1.030) in a large test tube or small Erlenmeyer flask add about 1 gm. of special bone black,[3] shake vigorously occasionally for 5–10 minutes, and filter through a small dry filter paper.

From 1 to 3 c.c. of the filtrate (if less than 3 c.c. are used, make up with distilled water to 3 c.c.) are measured into a test tube graduated at 25 c.c. (the tube employed for the blood sugar estimation is satisfactory, see p. 387) and 1 c.c. of 0.6 per cent picric acid and 0.5 c.c. of 5 per cent sodium hydroxide added.

When the tube is ready to be put in the boiling water bath, 5 drops of acetone solution[4] are added, care being taken that the acetone does not fall on the inside wall of the test tube. The solution is now well mixed, the tube closed with a cotton plug and boiled for 12–15 minutes. At the same time 3 c.c. of a solution containing 1 mg. of pure glucose are treated in a similar sugar tube with 1 c.c. of 0.6 per cent picric acid, 0.5 c.c. of 5 per cent sodium hydroxide, 5 drops of the acetone solution and then heated in the boiling water bath for the same length of time as the unknown.

Both tubes are now cooled to room temperature and made up to 25 c.c. and compared. If the unknown contains less than 0.7 mg. sugar it is better to have a standard containing 0.5 mg. of sugar and dilute both unknown and standard to 12.5 c.c.

Calculation.—$\frac{\text{Reading of standard}}{\text{Reading of unknown}}$ = mg. sugar in the 1, 2, or 3 c.c. of urine used. Correction must of course be made for any dilution of the original urine.

Interpretation.—The quantity of sugar in normal urine varies with the individual but is very small in any case. The maximum excretion is probably about 1.5 gram per day, a part only of this being fermentable.[5] Urines may be fermented with ordinary compressed yeast and sugar again determined. Fermentable and non-fermentable carbohydrate are thus estimated. The chief source of non-fermentable sugar may be protein metabolism.[6]

2. Determination of Sugar in Normal Urine. Method of Folin and Berglund.[7]—*Principle.*—The urine is shaken with "Lloyd's alkaloidal reagent," a concentrated fuller's earth, to remove interfering substances. Sugar is then determined as in blood filtrates, using the Folin-Wu colorimetric method.

Procedure.—To 5 c.c. of a concentrated urine add 5 c.c. N/10 sulphuric acid and 10 c.c. of water. Add 1.5 gram of Lloyd's reagent[8] and shake gently for 2 minutes

[1] Sumner's method p. 746 may also be used to determine sugar in normal urine.

[2] Benedict and Osterberg: *Jour. Biol. Chem.*, 48, 51, 1921.

[3] The special bone black may be prepared by treating 250 gm. of bone black with 1.5 liters of dilute hydrochloric acid (1 part of acid to 4 parts of water) and boiling for 30 minutes. The bone black is now filtered on a large Buchner funnel and washed with hot water until the filtrate is free from acid. Dry and powder.

[4] The acetone solution is prepared by mixing 1 part of C.P. acetone with 1 part of distilled water. The solution should be freshly prepared at frequent intervals.

[5] Kingsbury, (*Jour. Biol. Chem.*, 67, xviii, 1926) regards the physiological limit of urinary sugar as 0.2 %.

[6] Blatherwick, Bell, Hill and Long: *Jour. Biol. Chem.*, 66, 801, 1925.

[7] Folin and Berglund: *Jour. Biol. Chem.*, 51, 209, 1922. Folin's modification of the colorimetric method (see p. 384) may be employed.

[8] This may be obtained from Eli Lilly and Co., Indianapolis.

(not longer). Filter. Two c.c. of the filtrate are usually taken for the sugar determination according to the Folin-Wu technique (see Chapter on Blood Analysis). For more dilute urines take 10–15 c.c. and reduce water taken correspondingly.

For the determination of total sugar hydrolyze as follows: To 10 c.c. of the filtrate obtained after shaking with Lloyd's reagent add 1 c.c. of 10 per cent hydrochloric acid and heat in boiling water for 75 minutes. This heating should be done in test-tubes graduated at 20 c.c. for purposes of subsequent dilution. After hydrolysis, cool thoroughly and neutralize with normal sodium hydroxide. Phenolphthalein may be used as indicator, if desired, but is not necessary as the cloud produced from the material dissolved out of Lloyd's reagent furnishes an adequate indicator of the degree of neutrality required. Add the alkali until the cloud so formed does not disappear on shaking.

Dilute the neutralized hydrolysate to the 20 c.c. mark. Then add a small pinch of Lloyd's reagent and invert half a dozen times. Filter. This is for the purpose of removing most of the coloring matter formed during the hydrolysis. Two c.c. of this more dilute filtrate is usually a suitable amount to take for this determination also.

Interpretation.—According to the authors normal glycuresis represents, not glucose excretion, but the absorption and excretion of foreign unusable carbohydrate materials present in grains, vegetables and fruits, and decomposition products due to cooking, canning, and baking of such foods. An increase in carbohydrate material is noted after each meal.

3. Benedict's Copper Reduction Method for Sugar in Normal Urine.[1]—This method is the same in all respects as the method of Folin and Berglund (above) with the exception that new reagents[2] are substituted for the Folin-Wu copper and molybdic acid reagents. This method gives slightly lower results than the Folin-Berglund procedure.

Protein[3]

1. Folin's Gravimetric Method for the Determination of Protein in Urine.[4]
Principle.—The protein precipitated by heat and acetic acid is centrifuged, washed, dried and weighed.

Procedure.—Pipette 10 c.c. of urine into an ordinary conical centrifuge tube, which has been previously weighed; add 1 c.c. of 5 per cent acetic acid, and let stand for fifteen minutes in a beaker of boiling water. At the end of this time remove the tube from the water bath, and centrifuge for a few minutes. Pour off the supernatant liquid, stir up the precipitate in the tube with about 10 c.c. of boiling 0.5 per cent acetic acid, and again centrifuge. Remove the supernatant liquid from the precipitate in the tube, and wash once more, this time with 50 per cent alcohol. After centrifuging and pouring off the supernatant alcohol, place the tube for two hours in an air bath at 100 to 110°, then cool in a desiccator, and weigh.

The precipitate may also be filtered off and washed on a small tared filter paper then dried and weighed. In this case it is better to use a larger volume of

[1] Benedict: *Jour. Biol. Chem.*, 68, 759, 1926.
[2] These reagents are described on page 384.
[3] The method for albumin adopted by the Committee on Urinary Impairments of the Association of Life Insurance Medical Directors of America may be found on page 756.
[4] Folin: *Laboratory Manual of Biological Chemistry*, New York, Appleton, 1925.

urine. Instead of weighing, the nitrogen in the precipitate may be determined by the Kjeldahl method[1].

Calculation.—Multiply the weight of the precipitate in grams by 10 to obtain the percentage of protein present. If other than 10 c.c. of urine is used multiply by the appropriate volume factor.

Interpretation.—The amount of albumin occurring in the urine is not necessarily an index of the severity or type of the disorder giving rise to it. Hence no significant figures can be given. Normal human urine probably contains a trace of albumin which is too slight to be detected or determined by the usual procedures. The determination of albumin may be of assistance in following the course of kidney disturbances, but the results can be interpreted only in the light of other clinical findings.

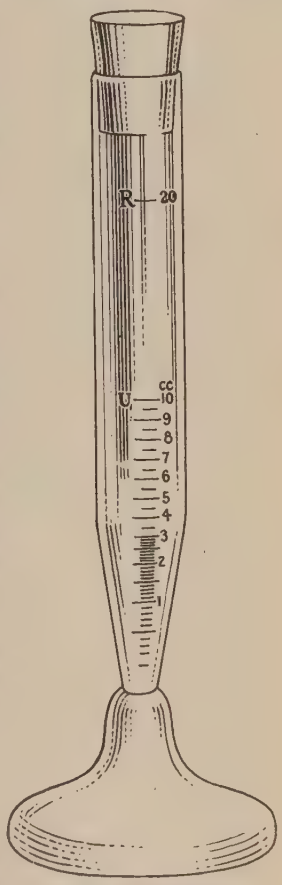


FIG. 230.—ESBACH'S ALBUMINOMETER.

2. **Esbach's Method.**—This method depends upon the precipitation of protein by Esbach's reagent[2] and the apparatus used in the estimation is Esbach's albuminometer (Fig. 230). In making a determination fill the albuminometer to the point U with urine, then introduce the reagent until the point R is reached. Now stopper the tube, invert it slowly several times in order to insure the thorough mixing of the fluids, and stand the tube aside for 24 hours. Creatinine, resin, acids, etc., are precipitated in this method, and for this and other reasons it is not as accurate as the coagulation method. It is, however, extensively used clinically. According to Sahli[3] the method is "accurate approximately to one part per 1000," whereas Pfeiffer[4] claims it is not accurate for less than one-half or for more than five parts per 1000. According to Quick[5] a 10 per cent solution of trichloroacetic acid is a better precipitant than Esbach's reagent.

Calculation.—The graduations on the albuminometer indicate grams of protein per liter of urine. Thus, if the protein precipitate is level with the figure 3 of the graduated scale, this denotes that the urine examined contains 3 grams of protein to the liter. To express the amount of protein in per cent simply move the decimal point one place to the left. In the case under consideration the urine contains 0.3 per cent protein.

[1] In order to arrive at correct figures for the protein content it is then simply necessary to multiply the total nitrogen content by 6.25 (see p. 546). Correction should be made for the nitrogen content of the filter paper used unless this factor is negligible.

[2] Esbach's reagent is prepared by dissolving 10 grams of picric acid and 20 grams of citric acid in 1 liter of water.

[3] Sahli: *Lehrbuch d. klin. Untersuchungs-Methoden*, 5th Aufl., 1909.

[4] Pfeiffer: *Berl. klin. Woch.*, 49, 114, 1912.

[5] Quick: *Jour. Lab. Clin. Med.*, 8, 1, 1923.

Interpretation.—See page 755.

3. Turbidity Method of Folin and Denis.[1]—*Principle.*—The albumin of the urine is precipitated with sulphosalicylic acid and the turbidity produced compared with that of a standard protein solution.

Procedure.—To about 75 c.c. of water in each of two 100 c.c. volumetric flasks is added 5 c.c. of a 25 per cent solution of sulphosalicylic acid. To one flask is then added 5 c.c. of the standard protein solution containing 10 mg. of albumin and to the other is added the albuminous urine 1 c.c. at a time (by means of an Ostwald pipette) until the turbidity obtained seems to be reasonably near that of the standard. The two flasks are then filled up to the mark with water, cautiously inverted a few times to secure mixing, and are then ready for the quantitative comparison just as in colorimetric work. The standard must invariably be read against itself. The standard[2] containing 10 mg. of protein is set at 20 mm. The unknown must not read less than 10 nor more than 30.

Calculation.—Dividing 200 by the product of the reading of the unknown and the number of cubic centimeters of urine taken gives the albumin in milligrams per cubic centimeter of urine. The albuminous suspensions must not be shaken but mixed very carefully. The method is fairly accurate and requires but a few minutes if a standard solution is at hand. The method is not applicable to urines deeply colored with blood or bile but may be used for albuminous fluids other than urine if such fluids are not highly pigmented. It must be borne in mind that different proteins, as serum albumin and serum globulin, may give markedly different degrees of turbidity under the same conditions.[3]

Interpretation.—See page 755.

4. The Determination of Albumin. Method adopted by the Committee on Urinary Impairments of the Association of Life Insurance Medical Directors of America.

Principle.—Clarified urine is treated with sulphosalicylic acid and the degree of turbidity produced, is measured by comparison with artificial standards.

Procedure.—Pipette 2.5 c.c. of centrifuged urine into a test tube graduated at 10 c.c. and add 3 per cent sulphosalicylic acid (30 grams dissolved and diluted to 1000 c.c. with distilled water) to the 10 c.c. mark. Invert the tube to mix, allow to stand 10 minutes and compare the turbidity with the permanent turbidity standards. Record the value of the standard most closely matched, as the albumin content of the urine.

Preparation of Permanent Albumin Standards.[4]**—20 grams of gelatin (Super X brand in sheet form, Coignet Chemical Products Company, New York) are**

[1] Folin and Denis: *Jour. Biol. Chem.*, 18, 273, 1914.

[2] Standard Albumin solution. *Fresh* blood serum free from hemoglobin is used 25–35 c.c. of the serum are diluted with a 15 per cent solution of chemically pure sodium chloride to about 1500 c.c. The solution is mixed and filtered. By means of nitrogen determinations the protein content of the filtrate is determined (protein = N × 6.25) and on the basis of the figure obtained the solution is diluted with 15 per cent sodium chloride solution so that it contains 2 mg. of protein per cubic centimeter. It is best to saturate the albumin solution with chloroform. The solution keeps for months.

[3] Marshall and Banks: Proceedings of the American Philosophical Society, 54, 176, 1915.

[4] Personal communication from Dr. Kingsbury, Chemist in Charge, Metropolitan Life Insurance Co. These permanent standards were developed in the laboratories of the Metropolitan Life Insurance Company and the Mutual Benefit Life Insurance Company. For a detailed description of the preparation of these standards, see Kingsbury, Clark, Williams and Post: *Jour. Lab. and Clin. Med.* 11, 981, 1926.

dissolved in 120 to 140 c.c. of distilled water at 45° to 55°C. and made up to 200 c.c. in a graduated cylinder. Approximately half the white of one egg is now added and stirred in. The mixture is then heated in a boiling water bath for at least 30 minutes after a temperature of 90°C. has been attained. It is filtered hot through a Whatman's No. 4 paper yielding a perfectly clear, slightly yellow solution. Immediately before using, 0.3 c.c. of formalin (40 per cent formaldehyde solution) is added to each 100 c.c. of gelatin solution. Formazin, the material to be suspended in the gelatin, is made as follows: 2.5 grams of urotropin are dissolved in 25 c.c. of distilled water at room temperature. This solution is added to 25 c.c. of hydrazine sulphate solution containing 0.25 grams of this substance also at room temperature. After mixing, the flask is stoppered and allowed to stand at least 15 hours. There will be a considerable quantity of a white amorphous material present which will be uniformly suspended on gently inverting the flask a few times. 14.5 c.c. of the formazin suspension is added to 100 c.c. of 10 per cent gelatin solution at 45°C. to 55°C. (to which the correct amount of formalin has previously been added) and thoroughly mixed. This produces a turbidity equivalent to that made by an albumin solution of 0.1 per cent, or one containing 100 milligrams of albumin in 100 c.c. when precipitated by 3 volumes of 3 per cent sulphosalicylic acid. This stock suspension is diluted as follows to make the remaining standards:

Stock formazin suspension equivalent to 100 mg. albumin per 100 c.c.	10% clarified gelatin	Value of standard made
c.c.	c.c.	
25.0	26	0.05 % or 50 mg.
20.0	30	0.04 % or 40 mg.
15.0	35	0.03 % or 30 mg.
10.0	40	0.02 % or 20 mg.
5.0	45	0.01 % or 10 mg.
2.5	55	0.005% or 5 mg.

Each standard is then poured into a test tube of the same dimensions as those used in making the test with urine. The tube is sealed with a waxed stopper and allowed to cool to room temperature. In a short time the gelatin should solidify and after a few days can not be melted at any room temperature. In extremely hot weather the tubes should be put in a cool place for a few days before any attempt is made to use them. The standards should be kept in a well lighted room but if in the course of time they take on a greenish tint, this may be bleached by exposure at a well lighted window without changing the turbidimetric value of the standard.

The standards may be checked by comparing them with sheep serum standards of known protein content precipitated by 3 volumes of sulphosalicylic acid in the same manner as in the urine test. Standards older than eight months should be replaced by new ones unless it is shown by actual test that they have still their original degree of turbidity, since it has been demonstrated that in the course of a year there may be a very slight diminution in the turbidimetric value of standards of this kind. There is no detectable change in six to eight months.

5. **Bence-Jones Protein. Method of Folin and Denis.**[1]—*Principle.*—A known volume of urine is heated at 60°, the coagulated Bence-Jones protein is then thrown down by centrifugation, washed with 50 per cent. alcohol, dried and weighed. The invariable presence of other proteins reduces the accuracy of any method for the determination of Bence-Jones protein.

Procedure.—Place 10 c.c. of the urine containing Bence-Jones protein in a previously weighed, conical centrifuge tube, add 1 c.c. of 5 per cent. acetic acid,[2] and allow to stand overnight[3] in a water bath at 60°C. The next morning remove the tube from the bath, centrifuge for a few minutes and pour off the supernatant liquid. Stir the sediment well with about 10 c.c. of 50 per cent. alcohol, centrifuge, pour off the alcohol, dry at 100°C. to constant weight, cool, and weigh.

Calculation.—Subtract the weight of the empty tube from that of the tube and protein to obtain the weight of Bence-Jones protein contained in 10 c.c. of urine. Calculate, from this figure, the percentage and 24-hour output.

Interpretation.—For a discussion of the significance of Bence-Jones proteinuria see p. 648.

Acetone Bodies

1. **Van Slyke's Methods.**[4]—*Principle.*—The method is based on a combination of Shaffer's oxidation of β-hydroxybutyric acid to acetone, (p. 762) and Denigè's precipitation of acetone as a basic mercuric sulphate compound. Glucose and certain other interfering substances are removed by precipitation with copper sulphate and calcium hydroxide. Preservatives other than toluene or copper sulphate should not be used.

Procedure.—Removal of Glucose and other Interfering Substances from Urine.

Place 25 c.c. of urine in a 250 c.c. measuring flask. Add 100 c.c. of water, 50 c.c. of copper sulphate solution[5] and mix. Then add 50 c.c. of 10 per cent calcium hydroxide suspension, shake, and test with litmus. If not alkaline, add more calcium hydroxide. Dilute to the mark and let stand at least one-half hour for glucose to precipitate. Filter through a dry folded filter. This procedure will remove up to 8 per cent of glucose. Urine containing more should be diluted

[1] Folin and Denis: *Jour. Biol. Chem.*, 18, 277, 1914.

[2] Taylor and Miller (*Jour. Biol. Chem.*, 25, 281, 1916) suggest the use of only a trace of acetic acid.

[3] From 6 P. M. until 8 A. M. is suggested as convenient.

[4] Van Slyke: *Jour. Biol. Chem.*, 32, 455, 1917.

[5] *Solutions Required.*—20 per cent copper sulphate—200 grams of $CuSO_4 \cdot 5H_2O$ dissolved in water and made up to 1 liter.

10 per cent mercuric sulphate—73 grams of pure red mercuric oxide dissolved in 1 liter of H_2SO_4 of 4 N. concentration.

50 volume per cent sulphuric acid—500 c.c. of sulphuric acid of 1.835 specific gravity, diluted to 1 liter with water. Concentration of H_2SO_4 must be readjusted if necessary to make it 17.0 N by titration.

10 per cent calcium hydroxide suspension—mix 100 grams of Merck's fine light "reagent" $Ca(OH)_2$ with 1 liter of water.

5 per cent potassium dichromate—50 grams $K_2Cr_2O_7$ dissolved in water and made up to 1 liter.

Combined reagents for total acetone body determination—1 liter of the above 50 per cent sulphuric acid, 3.5 liters of the mercuric sulphate, 10 liters of water.

enough to bring the glucose down to 8 per cent. The copper treatment is depended upon to remove interfering substances other than glucose, and should therefore never be omitted, even when glucose is absent. The filtrate may be tested for glucose by boiling a little in a test-tube. A precipitate of yellow cuprous oxide will be obtained if the removal has not been complete. A slight precipitate of white calcium salts always forms, but does not interfere with the detection of the yellow cuprous oxide.

Determination of Total Acetone Bodies (Acetone, Acetoacetic Acid, and β-Hydroxybutyric Acid.)—Place in a 500 c.c. Erlenmeyer flask 25 c.c. of urine filtrate. Add 100 c.c. of water, 10 c.c. of 50 per cent sulphuric acid, and 35 c.c. of the 10 per cent mercuric sulphate. Or in place of adding the water and reagents separately, add 145 c.c. of the "combined reagents." Connect the flask with a reflux condenser having a straight condensing tube of 8 or 10 mm. diameter and heat to boiling. After boiling has begun, add 5 c.c. of the 5 per cent dichromate through the condenser tube. Continue boiling gently 1 1/2 hours. The yellow precipitate which forms consists of the mercury sulphate-chromate compound[1] of the preformed acetone, and the acetone which has been formed by decomposition of acetoacetic acid and by oxidation of the β-hydroxybutyric acid. It is collected in a Gooch or "medium density" alundum crucible, washed with 200 c.c. of cold water, and dried for an hour at 110°. The crucible is allowed to cool in room air (a desiccator is unnecessary and undesirable) and weighed. Several precipitates may be collected, one above the other, without cleaning the crucible. As an alternative to weighing, the precipitate may be dissolved and titrated as described below.

Determination of Acetone and Acetoacetic Acid.—The acetone plus the acetoacetic acid, which completely decomposes into acetone and CO_2 on heating, is determined without the β-hydroxybutyric acid exactly as the total acetone bodies, except that (1) no dichromate is added to oxidize the β-hydroxybutyric acid and (2) the boiling must continue for not less than 30 nor more than 45 minutes. Boiling for more than 45 minutes splits off a little acetone from β-hydroxybutyric acid even in the absence of chromic acid.[2]

Determination of β-Hydroxybutyric Acid.—The β-hydroxybutyric acid alone is determined exactly as total acetone bodies except that the performed acetone and that from the acetoacetic acid are first boiled off. To do this the 25 c.c. of urine filtrate plus 100 c.c. of water are treated with 2 c.c. of the 50 per cent sulphuric acid and boiled in the open flask for 10 minutes. The volume of

[1] This contains about 77 per cent mercury and in the absence of chromate has approximately one of the following formulas: $3HgSO_4.5HgO.2[CH_3]_2CO$ or $2HgSO_4.3HgO.[CH_3]_2CO$.

[2] *Blank Determination of Precipitate from Substances in Urine Other than the Acetone Bodies.*—The 25 c.c aliquot of urine filtrate is treated with sulphuric acid and water and boiled 10 minutes to drive off acetone. The residue is made up to 175 c.c. with the same amounts of mercuric sulphate and sulphuric acid used in the above determinations, but without chromate, and is boiled under the reflux for 45 minutes. Longer boiling splits off some acetone from β-hydroxybutyric acid, and must therefore be avoided. The weight of precipitate obtained may be subtracted from that obtained in the above determination.

The blank is so small that it appears to be relatively significant only when compared with the small amounts of acetone bodies found in normal or nearly normal urines. In routine analyses of diabetic urines it is not determined.

Tests of Reagents.—When the complete total acetone bodies determination, including the preliminary copper sulphate treatment, is performed on a sample of distilled water instead of urine no precipitate whatever should be obtained. This test must not be omitted.

solution left in the flask is measured in a cylinder. The solution is returned to the flask, and the cylinder washed with enough water to replace that boiled off and restore the volume of the solution to 127 c.c. Then 8 c.c. of the 50 per cent sulphuric acid and 35 c.c. of mercuric sulphate are added. The flask is connected under the condenser and the determination is continued as described for total acetone bodies.

Titration of the Precipitate in the Above Methods.—Instead of weighing the precipitate, one may wash the contents of the Gooch, including the asbestos, into a small beaker with as little water as possible, and add 15 c.c. of normal HCl. The mixture is then heated, and the precipitate quickly dissolves. In case an alundum crucible is used, it is set into the beaker of acid until the precipitate dissolves, and then washed with suction, the washings being added to the beaker. In place of using either a Gooch or alundum crucible one may, when titration is employed, wash the precipitate without suction on a small quantitative filter paper, which is transferred with the precipitate to the beaker and broken up with a rod in 15 c.c. of normal HCl.

In order to obtain a good end-point in the subsequent titration it is necessary to reduce the acidity of the solution. For this purpose it has been found that the addition of excess sodium acetate is the most satisfactory means. Six to 7 c.c. of 3 M acetate are added to the cooled solution of redissolved precipitate. Then the 0.2 M KI is run in rapidly from a burette with constant stirring. If more than a small amount of mercury is present, a red precipitate of HgI_2 at once forms, and redissolves as soon as 2 or 3 c.c. of KI in excess of the amount required to form the soluble K_2HgI_4 have been added. If only a few mg. of mercury are present, the excess of KI may be added before the HgI_2 has had time to precipitate so that the titrated solution remains clear. In this case not less than 5 c.c. of the 0.2 M KI are added, as it has been found that the final titration is not satisfactory if less is present. The excess of KI is titrated back by adding 0.05 M $HgCl_2$ from another burette until a permanent red precipitate forms. Since the reaction utilized is $HgCl_2 + 4KI = K_2HgI_4 + KCl$, 1 c.c. of 0.05 M $HgCl_2$ is equivalent in the titration to 1 c.c. of the 0.2 M KI.

In preparing the two standard solutions the 0.05 M $HgCl_2$ is standardized by the sulphide method, and the iodine is standardized by titration against it. A slight error appears to be introduced if the iodide solution is gravimetrically standardized and used for checking the mercury solution, instead of vice versa.

In standardizing the mercuric chloride the following procedure has been found convenient: 25 c.c. of 0.05 M $HgCl_2$ are measured with a calibrated pipette, diluted to about 100 c.c., and H_2S is run in until the black precipitate flocculates and leaves a clear solution. The HgS, collected in a Gooch crucible and dried at 110°, should weigh 0.2908 gram if the solution is accurate.

Both by gravimetric analyses of the basic mercuric sulphate-acetone precipitate and by titration, the mercury content of the precipitate has been found to average 76.9 per cent. On this basis, each c.c. of 0.2 M KI solution, being equivalent to 10.0 mg. of Hg, is equivalent to 13.0 mg. of the mercury acetone precipitate.

Titration is not quite so accurate as weighing, but, except when the amounts determined are very small, the titration is satisfactory.

Calculation.—1 mg. of β-hydroxybutyric acid yields 8.45 mg. of precipitate. 1 mg. of acetone yields 20.0 mg. of precipitate. 1 c.c. of 0.2 M KI solution is equivalent to 13 mg. of precipitate in titration of the latter.

In order to calculate the acetone bodies as β-hydroxybutyric acid rather than acetone, use the above factors multiplied by the ratio of the molecular weights $\frac{\beta\text{-acid}}{\text{acetone}} = \frac{104}{58} = 1.793$. In order to calculate the acetone bodies in terms of molecular concentration, divide the factors in the table by 58. To calculate c.c. of 0.1 M acetone bodies per liter of urine use the above factors multiplied by $\frac{10{,}000}{58} = 172.4$.

Special Factors for Calculation of Results when 25 c.c. of Urine Filtrate, Equivalent to 2.5 c.c. of Urine, are used for the Determination.

Determination performed	Acetone bodies, calculated as gm. acetone per liter of urine, indicated by	
	1 gm. of prec.	1 c.c. of 0.2 M KI sol.
Total acetone bodies[1]	24.8	0.322
β-Hydroxybutyric acid	26.4	0.344
Acetone[2] + acetoacetic acid	20.0	0.260

Interpretation.—Normal adults on a mixed diet excrete on the average 3–15 mg. of combined acetone and acetoacetic acid per day and anything over 20 mg. is usually pathological. Usually about one-fourth of this total is acetone although the proportion varies considerably. The amount is considerably increased in fasting and on a carbohydrate-free diet due to the development of acidosis. In severe diabetic acidosis values up to 6 grams per day or even higher may be noted. It is sometimes found in large amounts in intoxications associated with pregnancy. It may be found in increased amounts in the urine in a great variety of pathological conditions. Quantative estimation enables us to follow the course of the acidosis. Ammonia excretion is also largely increased in these conditions, being used in the neutralization of the excess acids formed in the body. Usually about three-quarters of the combined acetone and acetoacetic acid excretion is in the form of acetoacetic acid, but the proportion is not constant.

[1] The "total acetone bodies" factor is calculated on the assumption that the molecular proportion of them in the form β-hydroxybutyric acid is 75 per cent of the total, which proportion is usually approximated in acetonuria. Because β-hydroxybutyric acid yields only 0.75 molecule of acetone, the factors are strictly accurate only when this proportion is present, but the error introduced by the use of the approximate factors is for ordinary purposes not serious. The actual errors in percentage of the amounts determined are as follows: molecular proportion of acetone bodies as β-acid 0.50, error 6.5 per cent; β-acid 90.0, error 3.8 per cent; β-acid 0.80, error 1.3 per cent.

[2] For the determination of preformed acetone see method p. 763.

β-Hydroxybutyric acid may occur in normal human urine to the extent of 20–30 mg. per day. In fasting or on a carbohydrate-free diet very large amounts may be excreted (up to 20 grams per day). In severe diabetes mellitus the largest amounts are found, and excretions of 50 or even 100 grams or over per day have been noted. In this condition it is usually the most abundant of the acetone bodies making up from 60–80 per cent of the total. The ratio is, however, by no means constant and it should be borne in mind that in rare cases large amounts of β-hydroxybutyric acid may be eliminated although the acetone excretion is very low. It is always present in the urine when large amounts of acetone are present.

A discussion of disturbance of acid-base equilibrium due to abnormal fat metabolism may be found in Chapter XIX.

2. Method of Shaffer and Marriott.[1]—*Principle.*—By this procedure the combined acetone and acetoacetic acid is determined in the same sample of urine used in the determination of β-hydroxybutyric acid. The preformed acetone and the acetoacetic acid are distilled off together as acetone and determined by the iodine titration method. The β-hydroxybutyric acid remains in the residue from distillation and is oxidized by means of potassium bichromate. The product of the oxidation is acetone which is distilled off and determined as such.

Procedure.—Determination of Acetone and Acetoacetic Acid.—Measure from 25–100 c.c.[2] or more of urine (usually) 50 c.c. with a pipette into a 500 c.c. volumetric flask containing 200–300 c.c. of water. Add basic lead acetate solution (U.S.P.) in volume equal to that of the urine used[3] and mix well. Add concentrated ammonium hydroxide in amount equal to about one-half that of the lead acetate solution. Dilute the contents of the flask to the mark with water, shake, and after standing a few minutes filter the liquid, preferably through a folded filter. Measure 200 c.c. of the filtrate into a round bottom flask (800 c.c. or liter Kjeldahl flasks are convenient) dilute with water to about 600 c.c. and add 15 c.c. of concentrated sulphuric acid and a little talc or a boiling stone. Distil until about 200 c.c. of distillate have been collected. The tube of the condenser should dip beneath the surface of the water in the receiving flask so that no loss of acetone will occur. The distilling flask must also be fitted with a dropping tube or dropping funnel so water may be run in from time to time and the volume of liquid in the flask kept from becoming less than 400–500 c.c. A good condenser should be used, but it is not necessary to cool the distillate in ice.

[1] Shaffer and Marriott: *Jour. Biol. Chem.*, 16, 265 1915.

[2] The amount used depends upon the expected yield of β-oxybutyric acid. In the case of urines which give a strong ferric chloride reaction for diacetic acid, or when 5–10 grams or more of β-oxybutyric acid is expected, it is unnecessary to use more than 25–50 c.c. of urine. However, in case only a trace of β-oxybutyric acid is expected, the volume should be much larger as indicated. Under all conditions, the amount specified is sufficient for duplicate determinations. It is desirable to use such a volume of urine as contains the proper amount of β-oxybutyric acid to yield 25–50 mg. of acetone.

[3] If the urine contains but little or no sugar only half the amount or less of lead acetate should be used.

The distillate thus obtained is transferred to a second Kjeldahl flask and 10 c.c. of 10 per cent NaOH added. It is then redistilled for about 20 minutes.[1] The second distillate is then titrated with standard iodine and thiosulphate solutions.

Calculation.—Subtract the number of cubic centimeters of N/10 thiosulphate solution used from the volume of N/10 iodine solution employed. Since 1 c.c. of the iodine solution is equivalent to 0.967 mg. of acetone, and since 1 c.c. of the thiosulphate solution is equivalent to 1 c.c. of the iodine solution, if we multiply the remainder from the above subtraction by 0.967 we will obtain the number of milligrams of acetone and acetoacetic acid, expressed as acetone, in the 100 c.c. of urine examined.

Determination of the β-Hydroxybutyric Acid.—The flask containing the residue from the first distillation above is used in the determination of the β-hydroxybutyric acid. A new receiver is arranged as before with the tip from the condenser dipping beneath the surface of the water. The distillation is then continued and water added whenever necessary to keep the volume between 400 and 600 c.c. A dilute solution (1 per cent) of potassium bichromate is added during the distillation. At first 20 c.c. of this 1 per cent solution is added slowly through the dropping tube and then 10 c.c. portions every 15–20 minutes until the whole has been added.[2] Should the liquid become markedly green the bichromate must be added at correspondingly shorter intervals and in amount sufficient to maintain a slight red-yellow color of the chromic acid which may be detected even in the presence of the green. Continue the distillation with moderate boiling for from two to three hours. The distillate which should be collected in a liter flask to avoid transference is again distilled for about 20 minutes after adding 10 c.c. of 10 per cent sodium hydroxide and 25 c.c. of 3 per cent hydrogen peroxide. The flask must be heated cautiously until the peroxide has been decomposed. This final distillate is titrated with standard iodine and thiosulphate solutions in the usual manner and the result expressed as hydroxybutyric acid. One c.c. of N/10 iodine solution is equivalent to 1.736 mg. of hydroxybutyric acid. One c.c. of the 1.035 N/10 iodine which is recommended for acetone titrations is equivalent to 1.793 mg. of hydroxybutyric acid. About 10 per cent should be added to the results for β-hydroxybutyric acid as obtained by this method as the yield of acetone is only about 90 per cent of the theoretical. This error appears to be practically constant, so that satisfactory results may be obtained by correction.

Acetone

Folin's Method.—*Principle.*—The preformed acetone is aspirated from the urine mixture at room temperature to prevent decomposition of acetoacetic acid. The acetone is collected in alkaline hypoiodite solution as in the Folin-Hart method. Iodoform is formed

[1] In many instances when a high degree of accuracy is not required this redistillation may be omitted and the first distillate titrated directly. The results so obtained are slightly higher than those after redistillation from alkali. The object of the redistillation is to get rid of fatty acids of which formic acid is one of the most troublesome.

[2] From 0.5 gram to 1 gram of bichromate will usually be sufficient, and not more than 1 gram should be added unless the liquid turns green indicating a great reduction to chromium sulphate. Very rarely 2 or 3 grams of bichromate may be necessary, especially if the sugar has not been completely removed.

quantitatively and the excess of iodine is titrated with sodium thiosulphate.

Procedure.—The same type of apparatus is used in this method as that described in Folin's method for the determination of ammonia (see page 724). Introduce 20–25 c.c. of the urine under examination into the aerometer cylinder and add 10 drops of 10 per cent phosphoric acid,[1] 8–10 grams of sodium chloride,[2] and a little petroleum. Introduce into an absorption flask,[3] such as is used in the ammonia determination (see page 725), 150 c.c. of water, 10 c.c. of a 40 per cent solution of potassium hydroxide, and an excess of a N/10 iodine solution. Connect the flask with the aerometer cylinder, attach a Chapman pump, and permit an air current, slightly less rapid than that used for the determination of ammonia, to be drawn through the solution for 20–25 minutes. All of the acetone will, at this point, have been converted into iodoform in the absorption flask. Add 10 c.c. of concentrated hydrochloric acid (a volume equivalent to that of the strong alkali originally added), to the contents of the latter and titrate the excess of iodine by means of N/10 sodium thiosulphate solution until a light yellow color is obtained. At this point add a few cubic centimeters of starch paste and titrate the mixture until no blue color is visible. This is the end reaction.

Calculation.—Subtract the number of cubic centimeters of N/10 thiosulphate solution used from the volume of N/10 iodine solution employed. Since 1 c.c. of the iodine solution is equivalent to 0.967 mg. of acetone, and since 1 c.c. of the thiosulphate solution is equivalent to 1 c.c. of the iodine solution, if we multiply the remainder from the above subtraction by 0.967 we will obtain the number of milligrams of acetone in the volume of urine employed.

Calculate the quantity of acetone in the twenty-four-hour urine specimen.

Interpretation.—See Van Slyke's Methods, page 761.

Folin has further made suggestions regarding the *simultaneous* determination of acetone and ammonia by the use of the same air current.[4] This is an important consideration for the clinician inasmuch as urines which contain acetone and acetoacetic acid are generally those from which the ammonia data are also desired. The procedure for the combination method is as follows: Arrange the ammonia apparatus as usual (see page 725), and to the aerometer of the ammonia apparatus attach the acetone apparatus set up as described above. Regulate the air current with special reference to the determination of acetone and at the end of 20–25 minutes disconnect the acetone apparatus and complete the determination of the acetone as just described. The air current is not interrupted, and after having run one and one-half hours the ammonia apparatus is detached and the ammonia determination completed as described on page 725.

If data regarding acetoacetic acid are desired, the result obtained by Folin's method may be subtracted from the result obtained by the Van Slyke method

[1] Oxalic acid (0.2—0.3 gram) may be substituted if desired.

[2] Acetone is insoluble in a saturated solution of sodium chloride.

[3] Folin's improved absorption tube (see Fig. 228, p. 725) should be used in this connection inasmuch as the original type embracing the use of a rubber stopper is unsatisfactory because of the solvent action of alkaline hypoiodite on rubber.

[4] These determinations may even be made on the *same sample* of urine if the sample is too small for the double determination.

for acetone and acetoacetic acid. Under all conditions the determination of acetone should be as expeditious as possible. This is essential, not only because of the fact that any acetoacetic acid present in the urine will become transformed into acetone, but *also* because of the rapid spontaneous decomposition of the alkaline hypoiodite solution used in the determination of the acetone. It has been claimed that alkaline hypoiodite solutions are almost completely converted into *iodate* solutions in *one-half hour*. Folin states, however, that the transformation is not so rapid as this, but he nevertheless emphasizes the necessity of rapidity of manipulation. At the same time it should be remembered that the air current must not be as rapid as for ammonia, inasmuch as the alkaline hypoiodite solution will not absorb all the acetone under those conditions.

Indican

1. Parker's Modification of Method of Askenstedt.[1] *Principle.*—Interfering substances are precipitated with mercuric chloride. Indican is converted to indigo by treatment with Obermayer's reagent and extracted with chloroform. The chloroform extract is diluted with alcohol and the blue color compared with that of a standard solution of indigo. Ellinger has devised a titration method.[2] Plasencia[3] has suggested a method which is shorter than Ellinger's and according to its sponsor, just as accurate.

Procedure.—Saturate 35 c.c. of urine with solid mercuric chloride, let stand for half an hour, and filter. Heat two 10 c.c. portions of filtrate to 48–50°C. Add to one 10 c.c. of Obermayer's reagent, mix and add at once 8 c.c. of chloroform. To the other tube (to be used as a standard) add 10 c.c. of concentrated HCl and 8 c.c. of chloroform. Extract for two minutes. Pour off supernatant fluids. Wash the extracts twice by shaking with distilled water. Pour off as much water as possible. Add to each enough of a 0.1 per cent solution of mercuric chloride in 95 per cent alcohol to make a volume of 25 c.c. To the standard tube now add from a burette enough of a solution of C. P. indigo (1 c.c. = 1 mg.) in concentrated sulphuric acid to produce the same color as in the unknown. If desired a colorimeter may be used. If the unknown has a greenish cast a drop of very dilute picric acid solution added to the standard is helpful. The alcoholic solutions fade rapidly especially in sunlight. Comparisons should therefore be made at once. The indigo solution should be kept in a brown glass stoppered bottle in a dark place.

Calculation.—C.c. of standard indigo solution = mg. indigo from 10 c.c. of urine.

Interpretation.—From 4–20 mg. of indican are excreted per day by normal men. In normal individuals the variations are dependent mainly upon the diet. A meat diet increases the indican excretion, while a milk or carbohydrate-rich diet decreases it. Pathologically the greatest increases are found in disorders involving increased putrefaction and stagnation of intestinal contents. Bacterial decomposition of body protein as in gangrene, putrid pus formation, etc., gives rise to increases.

[1] Askenstedt: *N. Y. Med. Jour.*, 95, 1363, 1912; Parker: Private communication, 1926.
[2] Hawk: Practical Physiological Chemistry, Eighth Edition, p. 568.
[3] Plasencia: *Revista de Medicina y Cirugia*, 17, 1, 1912.

Phenols

1. **Colorimetric Method of Folin and Denis.**[1]—*Principle.*—This method is based upon the fact that phenols yield with a solution of phosphotungstic-phosphomolybdic acid and alkali a deep blue color the depth of which is proportional to the amount of such substances present. Traces of protein, which may be present in the urine, and uric acid give a blue color with the reagent and are removed by precipitation with an acid silver lactate solution and colloidal iron as a preliminary to the determination of the phenols.

Procedure.—Removal of Interfering Substances.—Place 10 c.c. of ordinary urine, or 20 c.c. of a dilute urine in a 50 c.c. volumetric flask. To this add an acid silver lactate solution (from 2 to 20 c.c. of a 3 per cent solution of silver lactate in 3 per cent lactic acid) until no further precipitate is obtained. Add a few drops of colloidal iron, shake the flask, dilute to mark with distilled water, shake again, and filter the contents through a dry filter. Phenols are not precipitated by this procedure but are recovered quantitatively in the filtrate. Transfer 25 c.c. of the filtrate to a 50 c.c. volumetric flask, and add a sufficient quantity of saturated sodium chloride solution, containing 10 c.c. of strong hydrochloric acid per liter, to precipitate all the silver. Fill the flask to the mark with distilled water, mix thoroughly, and filter through a dry filter. This filtrate, which contains half the phenol from the urine taken for analysis, is used for the determination of free and total phenols.

Free Phenols.—Place 20 c.c. of the filtrate mentioned above in a 50 c.c. volumetric flask, add 5 c.c. of the phosphotungstic-phosphomolybdic acid reagent[2] and 15 c.c. of a saturated solution of sodium carbonate. Dilute to volume with luke warm water (30–35°C.), mix thoroughly and after allowing to stand for 20 minutes compare the deep blue color in the Duboscq colorimeter (see Fig. 110, page 361) against a standard solution of phenol (see below) similarly treated.

Total Phenols (Free and Conjugated).—Place 20 c.c. of the same filtrate used for the determination of free phenols in a large test-tube, add 10 drops of concentrated hydrochloric acid, cover the tube with a small funnel, heat rapidly to boiling over a free flame, and then place in a boiling water-bath for ten minutes. This process serves to decompose the conjugated phenols. At the end of the ten minutes, remove the tube, cool, and transfer the contents to a 100 c.c. volumetric flask. Add 10 c.c. of the phosphotungstic-phosphomolybdic reagent, 25 c.c. of saturated sodium carbonate solution, dilute to mark with luke warm water (30–35°C.), mix thoroughly, allow to stand for 20 minutes, and read in the Duboscq colorimeter (see page 361) against a standard solution of phenol (see below).

Standard Solution of Phenol.—The standard used is a solution of pure phenol in N/100 hydrochloric acid containing 1 mg. of phenol in 10 c.c., standardized by

[1] Folin and Denis: *J. Biol. Chem.*, 22, 305, 1915.

[2] This reagent is prepared as follows: Boil together for two hours (using a reflux condenser) 100 grams of sodium tungstate, 20 grams of phosphomolybdic acid (or an equivalent of molybdic acid), 50 c.c. of phosphoric acid (85 per cent), and 750 c.c. of distilled water. After the period of heating, cool, dilute to 1 liter with distilled water, and filter if necessary.

means of the iodometric titration. The preparation is carried out as follows: Make a phenol solution in N/10 hydrochloric acid, which contains approximately 1 mg. of crystallized phenol per cubic centimeter. Transfer 25 c.c. of this solution to a 250 c.c. flask, add 50 c.c. of N/10 sodium hydroxide, heat to 65°C., add 25 c.c. of N/10 iodine solution, stopper the flask, and let stand at room temperature 30 or 40 minutes. Add 5 c.c. of concentrated hydrochloric acid and titrate the excess of iodine with N/10 thiosulphate solution. Each cubic centimeter of N/10 iodine solution corresponds to 1.567 mg. of phenol. On the basis of the result dilute the phenol solution so that 10 c.c. contain 1 mg. of phenol. Five c.c. of this solution (equivalent to 0.5 mg. of phenol), when 10 c.c. of the phosphotungstic phosphomolybdic reagent and 25 c.c. of saturated sodium carbonate solution are added, and the whole made up with water at about 30°C. to 100 c.c., give when set in the colorimeter at 20 mm. a convenient standard.

Calculation.—The filtrate used for the determination of free and total phenols contains the phenols from one-half the amount of urine analyzed. The actual determination of phenols, both free and total, is made upon a two-fifths portion of this filtrate and this amount of filtrate contains the phenols from one-fifth of the amount of urine analyzed. In the determination of free phenols the colored solution is diluted to only half that of the standard while in the determination of total phenols the dilution is the same as that of the standard.

Hence,

$$\frac{R_1}{R_2 \times 4} = \text{milligrams of free phenol}$$

and

$$\frac{R_1}{R_2 \times 2} = \text{milligrams of total phenol}$$

in 2 c.c. or 4 c.c. of urine according to whether 10 c.c. or 20 c.c. of urine was taken for analysis, when R_1 is taken as the reading obtained with the standard solution, and R_2 is taken as the reading obtained with the unknown.

Interpretation.—This method determines all phenolic substances *e.g.*, the volatile phenols *p-cresol* and *phenol*, the non-volatile phenol *pyrocatechol* and the aromatic oxyacids *p-oxyphenylacetic acid*, *p-oxyphenylpropionic acid* and *p-oxybenzoic acid*. All these substances are formed from tyrosine. By this method total phenol excretions of from 0.2–0.5 gram per day have been noted in normal individuals. These results are much higher than figures previously obtained by other methods. The free phenols varied from 0.1–0.3 gram per day. The total phenol excretion appears to vary directly but not proportionately with the protein intake. The amount of conjugated phenol indicates the extent to which the phenols have been detoxicated. The excretion of phenols is increased in gastro-intestinal disorders associated with increased putrefaction. It is increased by the ingestion of phenols or of benzene. Constipation influences the phenol output to a greater extent than diet.[1] Diets which promote the growth of putrefactive

[1] Underhill and Simpson: *Jour. Biol. Chem.*, 44, 69, 1920.

bacteria also promote indican and phenol excretion. A high phenol output does not necessarily mean a high indican excretion.

2. **Tisdall's Modification of Folin-Denis Method.**[1]—*Principle.*—The phenolic substances are extracted from the urine with ether, the ether solution extracted with 10 per cent NaOH, and the phenols determined colorimetrically as in Folin-Denis method p. 766.

Procedure for Free Volatile Phenols.—Five c.c. of urine are shaken for five minutes with 100 c.c. of ether. The urine is separated and two more extractions are made, using 50 c.c. of ether each time. The 200 c.c. of ether are shaken for 5 minutes with 20 c.c. of 10 per cent NaOH, separated, and the sodium hydroxide solution is neutralized and made slightly acid with concentrated HCl. Sodium carbonate and the phenol reagent are then added as in the Folin-Denis method p. 766.

Procedure for Total Volatile Phenols.—The second filtrate from the Folin-Denis procedure is treated as outlined by Folin and Denis for the deconjugation of phenols and then extracted with ether as in Tisdall's procedure for free volatile phenols (see above).

Interpretation.—Results for total phenolic substances obtained by this method are at least 50 per cent lower than those recorded by Folin and Denis. By the Tisdall method only a small fraction of the volatile phenols are found to be excreted in the free state. The substances causing the difference in results by the two methods remain undetermined. For additional notes on interpretation see Folin-Denis method p. 767.

Urobilinogen

Method of Wallace and Diamond.[2]—*Principle.*—A series of dilutions of urine is carried to the point where the red color resulting from the reaction between urobilinogen and Ehrlich's aldehyde reagent is just discernible.

Procedure.—One c.c. of Ehrlich's aldehyde reagent[3] is added to 10 c.c. of undiluted urine and allowed to stand 1 to 3 minutes. An idea as to the quantity of urobilinogen is gained by noting the rapidity and intensity of color development. Dilutions are not carried out if the color remains a light red (normal values). For higher concentrations, prepare a series of dilutions of the urine from 1 : 10 to 1 : 200 or higher, as indicated by the preliminary test. Tap water may be used, but should not be too cold. Add one c.c. of the reagent to each dilution and after 3 to 5 minutes note the highest dilution that shows a faint pink discoloration. Express the result in terms of this dilution. The test is best performed in daylight but in the absence of bright sunlight.

[1] Tisdall: *Jour. Biol. Chem.*, 44, 409, 1920.

[2] Wallace and Diamond, *Arch. Int. Med.* 35, 698, 1925. This test is regarded as more delicate than the spectroscopic method of Wilbur and Addis, *Arch. Int. Med.*, 13, 235, 1914. A more extended, and probably more accurate method for the determination of urobilin and urobilinogen in urine and feces, is described by Terwen, *Nederland. Tijdschr. Geneeskunde*, 69, I, 2492, 1925. See also *Ibid.* 69, I, 2508, 2648, 1925.

[3] Prepared by dissolving 2 gm. paradimethylamidobenzaldehyde in 100 c.c. of 20 per cent hydrochloric acid.

Interpretation.—The appearance of the color in dilutions up to 1:20 may be regarded as normal. Constipation may produce a temporary rise in urobilinogen. This substance is believed to originate in the intestinal canal by reduction of the bile pigment. The latter may be extra-hepatic as well as hepatic in origin (see p. 398). Excessive amounts of urobilinogen are, therefore, excreted in diseases of the liver and biliary tract, including toxemias of pregnancy, infectious diseases and alcoholic intoxication, and in hemolytic diseases, including poisoning by lead, sulphonal, mushrooms and hemolytic poisons in general.

Oxalic Acid

Dakin Modification of Salkowski-Autenrieth and Barth Method.[1]—*Principle.* The oxalic acid is precipitated by means of $CaCl_2$. From the solution of this precipitate in hydrochloric acid the oxalic acid is extracted with ether and reprecipitated as calcium oxalate.

Procedure.—To a twenty-four hour specimen of urine (or as large a quantity as is available) add HCl to make about a 5 per cent solution and heat on the water bath for 20 minutes to break up oxaluric acid. Add an excess of calcium chloride and make fairly strongly alkaline with ammonia. Let stand in a warm place over night. Filter off on a folded filter and wash precipitate well with boiling water. Observe the filtrate for a short time. If calcium oxalate sediments filter again. Transfer precipitates to a beaker and warm with a small quantity of dilute HCl. Filter through a small folded filter. Wash the residue by digesting with successive portions of hot water. Concentrate filtrate to 5–7 c.c. Transfer to an extraction tube for continuous extraction with ether (see Fig. 231), and extract for 5–6 hours. Add 20 c.c. of water to the ether extract and distil off ether. Filter and add an excess of calcium chloride and ammonia. Then make decidedly acid with acetic acid and let stand in a warm place over night. Filter off precipitate on a small paper, and wash with hot water. Titrate with potassium permanganate after treating with an excess of 5 per cent sulphuric acid and warming to 60°, or ignite to CaO and weigh.

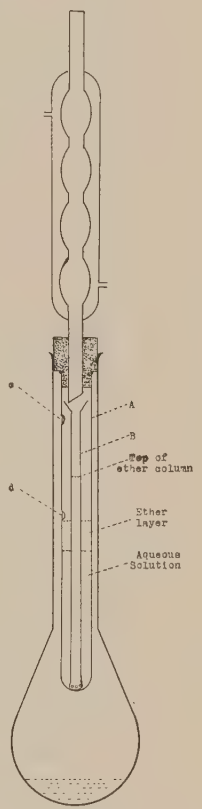


FIG. 231.—CONTINUOUS EXTRACTION APPARATUS (*Gaebler*).[2]

[1] Dakin: *Jour. Biol. Chem.*, 3, 57, 1907.

[2] As used by Dr. O. H. Gaebler, University of Iowa.

The apparatus involves essentially the same principles as the one used by Myers and Wardell in the determination of cholesterol (p. 392). Of the apparatus as shown in the diagram, only two parts need be made—tubes A and B. A is a medium weight Pyrex, 1 × 10 inch tube, with two holes, c and d, the former 8½ and the latter 5½ inches from the bottom. Tube B is made from ordinary glass tubing, is 9 inches in length, and has a funnel shaped dilation at the top and bulb with four or five small holes at the bottom. A cork which fits an 800 c.c. Kjeldahl flask is fitted with a hole to admit a small condenser, and is cut down to one inch diameter at its lower end so that tube A can be forced upon it. On heating, the ether vapor passes through c into the condenser, runs back through tube B, rises through the aqueous solution, and returns to the Kjeldahl flask through d.

Calculation.—Since 56 parts of CaO are equivalent to 90 parts of oxalic acid, the quantity of oxalic acid in the volume of urine taken may be determined by multiplying the weight of CaO by the factor of 1.6071. In case titration is performed it should be remembered that 1 c.c. N/10 $KMNO_4$ equals 0.0063 gm. crystallized oxalic acid or 0.0045 gm. anhydrous acid.

Interpretation.—From 15–20 mg. of oxalic acid are excreted by a normal adult on an ordinary mixed diet. It arises from oxalates of the food ingested and from fat and protein metabolism. It is increased by the ingestion of apples, grapes, cabbage, etc., although most of the ingested oxalate is destroyed. It is increased in disturbances of metabolism associated with decreased oxidation, according to certain observers. The term "oxaluria" has been largely a misnomer.

Sulphur

(a) Gravimetric Procedures

1. Total Sulphates.—*Folin's Method.*—*Principle.*—The sulphuric acid of the conjugated sulphates is set free by boiling with acid. The total sulphates are then precipitated with barium chloride.

Procedure.—Place 25 c.c. of urine in a 200-250 c.c. Erlenmeyer flask, add 20 c.c. of dilute hydrochloric acid[1] (1 volume of concentrated HCl to 4 volumes of water) and gently boil the mixture for 20-30 minutes. To minimize the loss of water by evaporation the mouth of the flask should be covered with a small watch glass during the boiling process. Cool the flask for 2-3 minutes in running water, and dilute the contents to about 150 c.c. by means of cold water. Add 10 c.c. of a 5 per cent solution of barium chloride slowly, drop by drop, to the cold solution.[2] The contents of the flask should not be stirred or shaken during the addition of the barium chloride. Allow the mixture to stand at least one hour, then shake up the solution and filter it through a weighed Gooch crucible.[3]

Wash the precipitate of $BaSO_4$ with about 250 c.c. of cold water, dry it in an air-bath or over a very low flame, then ignite,[4] cool and weigh.

Calculation.—Subtract the weight of the Gooch crucible from the weight of the crucible and the $BaSO_4$ precipitate to obtain the weight of the precipitate.

[1] If it is desired, 50 c.c. of urine and 4 c.c. of concentrated acid may be used instead.

[2] A dropper or capillary funnel made from an ordinary calcium chloride tube and so constructed as to deliver 10 c.c. in 2–3 minutes is recommended for use in adding the barium chloride.

[3] If a Gooch crucible is not available, the precipitate of $BaSO_4$ may be filtered off upon a washed filter paper (Schleicher & Schüll's, No. 589, blue ribbon), and after washing the precipitate with about 250 c.c. of *cold* water the paper and precipitate may be dried in an air-bath or over a low flame. The ignition may then be carried out in the usual way in the ordinary platinum or porcelain crucible. In this case correction must be made for the weight of the ash of the filter paper used.

[4] Care must be taken in the ignition of precipitates in Gooch crucibles. The flame should never be applied directly to the *perforated* bottom or to the sides of the crucible, since such manipulation is invariably attended by mechanical losses. The crucibles should always be provided with *lids* and *tight bottoms* during the ignition. In case porcelain Gooch crucibles, whose bottoms are not provided with a non-perforated cap, are used, the crucible may be placed upon the lid of an ordinary platinum crucible during ignition. The lid should be supported on a triangle, the crucible placed upon the lid and the flame applied to the improvised bottom. Ignition should be complete in 10 minutes if no organic matter is present.

The weight of SO_3 in the volume of urine taken[1] may be determined by means of the following proportion.

$$\underset{BaSO_4}{\text{Mol. Wt.}} : \underset{BaSO_4}{\text{Wt. of}} :: \underset{SO_3}{\text{Mol. wt.}} : x(\text{wt. of } SO_3 \text{ in grams}).$$

Representing the weight of the $BaSO_4$ precipitate by y and substituting the proper molecular weights, we have the following proportion:

233.43 : y : : 80.06 : x (wt. of SO_3 in grams in the quantity of urine used).

Calculate the quantity of SO_3 in the twenty-four-hour specimen of urine.

To express the result in percentage of SO_3 simply divide the value of x, as just determined, by the quantity of urine used.

Interpretation.—The total sulphate excretion (ethereal and inorganic sulphates) by a normal adult on a mixed diet is usually between 1.5 and 3.0 gram of SO_3 with an average of about 2.0 gram. The sulphuric acid is derived but to a slight extent ordinarily from ingested sulphates, being mainly dependent on the sulphur of the protein ingested and will consequently vary widely with the protein content of the diet. From 75 to 95 per cent of the total sulphur of the urine is ordinarily found as sulphate, the proportion being greatest on a high protein diet. The sulphate excretion is increased in all conditions associated with increased decomposition of body protein as in acute fevers and decreased whenever there is a decrease in metabolic activity.

2. Inorganic Sulphates.—Folin's Method.—Place 25 c.c. of urine and 100 c.c. of water in a 200–250 c.c. Erlenmeyer flask and acidify the diluted urine with 10 c.c. of dilute hydrochloric acid (1 volume of concentrated HCl to 4 volumes of water). In case the urine is dilute 50 c.c. may be used instead of 25 c.c. and the volume of water reduced proportionately. Add 10 c.c. of 5 per cent barium chloride slowly, drop by drop, to the cold solution and from this point proceed as indicated in the method for the determination of Total Sulphates, page 770.

Calculate the quantity of inorganic sulphates, expressed as SO_3, in the twenty-four-hour urine specimen.

Calculation.—Calculate according to the directions given under Total Sulphates, above.

Interpretation.—On an average about 90 per cent of the total sulphates of the urine exists as inorganic sulphates but the proportion of the sulphates existing in this form varies widely, being greater on a high protein diet than on a very low protein diet. The amount varies with the total sulphates (see above).

3. Ethereal Sulphates.—Folin's Method.—Principle.—The inorganic sulphates are removed with barium chloride and the conjugated sulphates then determined after hydrolysis.

[1] It is considered preferable by many investigators to express all sulphur values in terms of S rather than SO_3.

Procedure.—Place 125 c.c. of urine in an Erlenmeyer flask of suitable size, dilute it with 75 c.c. of water and acidify the mixture with 30 c.c. of dilute hydrochloric acid (1 volume of concentrated HCl to 4 volumes of water). To the cold solution add 20 c.c. of a 5 per cent solution of barium chloride, drop by drop.[1] Allow the mixture to stand about one hour, then filter it through a dry filter paper.[2] Collect 125 c.c. of the filtrate and boil it gently for at least one-half hour. Cool the solution, filter off the precipitate of $BaSO_4$, wash, dry and ignite it according to the directions given on page 770.

Calculation.—The weight of the $BaSO_4$ precipitate should be multiplied by 2 since only one-half (125 c.c.) of the total volume (250 c.c.) of fluid was precipitated by the barium chloride. The remaining calculation should be made according to directions given under Total Sulphates, page 770.

Calculate the quantity of ethereal sulphates, expressed as SO_3, in the twenty-four-hour urine specimen.

Interpretation.—The excretion of ethereal sulphates (expressed as SO_3) varies ordinarily from 0.1 to 0.25 gram per day comprising from 5 to 15 per cent of the total sulphur excretion. The absolute amount of ethereal sulphate increases with increase in the protein of the diet and particularly with increase of putrefactive processes in the intestine or elsewhere. The amount excreted cannot however be taken as an index of the extent of intestinal putrefaction.

4. Total Sulphur.[3]—Benedict's Method.[4]—*Principle.*—The urine is evaporated and ignited with a solution of copper nitrate and potassium chlorate. Organic matter is thus destroyed and all unoxidized sulphur is oxidized to the sulphate form and can be readily precipitated with barium chloride in the usual manner. The method is very convenient and accurate.

Ten c.c. of urine are measured into a small (7–8 cm.) porcelain evaporating dish and 5 c.c.[5] of Benedict's sulphur reagent[6] added. The contents of the dish are

[1] See note (2) at the bottom of p. 770.

[2] This precipitate consists of the inorganic sulphates. If it is desired, this $BaSO_4$ precipitate may be collected in a Gooch crucible or on an ordinary quantitative filter paper and a determination of inorganic sulphates made, using the same technic as that suggested above. In this way we are enabled to determine the inorganic and ethereal sulphates in the same sample of urine.

[3] For determination of total sulphur in other biological material see method by Stockholm and Koch: *Jour. Am. Chem. Soc.*, 45, 1953, 1923.

[4] Benedict: *Journal of Biological Chemistry*, 6, 363, 1909.

[5] If the urine is concentrated the quantity should be slightly increased.

[6] Crystallized copper nitrate, sulphur-free or of known sulphur content.....200 grams.
Sodium or potassium chlorate..... 50 grams.
Distilled water to..... 1000 c.c.

Denis has suggested the use of the following solution:

Copper nitrate	25 grams.
Sodium chloride	25 grams.
Ammonium nitrate	10 grams.
Water to make	100 c.c.

The procedure is the same as the above except that 25 c.c. of urine and 5 c.c. of reagent are taken. It gives accurate results.

evaporated over a free flame which is regulated to keep the solution just below the boiling-point, so that there can be no loss through spattering. When dryness is reached the flame is raised slightly until the entire residue has blackened. The flame is then turned up in two stages to the full heat of the bunsen burner and the contents of the dish thus heated to redness for ten minutes after the black residue (which first fuses) has become dry. This heating is to decompose the last traces of nitrate (and chlorate). The flame is then removed and the dish allowed to cool more or less completely. Ten to 20 c.c. of dilute (1 : 4) hydrochloric acid is then added to the residue in the dish, which is then warmed gently until the contents have completely dissolved and a perfectly clear, sparkling solution is obtained. This dissolving of the residue requires scarcely two minutes. With the aid of a stirring rod the solution is washed into a small Erlenmeyer flask,[1] diluted with cold, distilled water to 100–150 c.c., 10 c.c. of 10 per cent barium chloride solution added drop by drop, and the solution allowed to stand for about an hour. It is then shaken up and filtered as usual through a weighted Gooch crucible. Controls should be run on the oxidizing mixture.

Calculation.—Make the calculation according to directions given under Total Sulphates, page 770. Calculate the quantity of sulphur expressed as SO_3 or S, present in the twenty-four-hour urine specimen.

Interpretation.—The total sulphur [SO_3] excretion averages about 2.5 grams per day. It runs more or less parallel with the decomposition of endogenous and exogenous protein and a definite ratio between the excretion of total N and total S might be expected. It has been suggested that the ratio 5 : 1 expresses this relation in a general way but no constant value can be given. See Total Sulphates.

5. Total Sulphur.—Osborne-Folin Method.—*Principle.*—This method depends on the destruction of organic matter by means of sodium peroxide. It is employed particularly for the determination of sulphur in foods and feces. Benedict's procedure (see above) is simpler and fully as satisfactory for urine.

Place 25 c.c. of urine[2] in a 200–250 c.c. nickel crucible and add about 3 grams of sodium peroxide. Evaporate the mixture to a syrup upon a steam water-bath and heat it carefully over an alcohol flame until it solidifies (15 minutes). Now remove the crucible from the flame and allow it to cool. Moisten the residue with 1–2 c.c. of water,[3] sprinkle about 7–8 grams of sodium peroxide over the contents of the crucible and fuse the mass over an alcohol flame for about 10 minutes. Allow the crucible to cool for a few minutes, add about 100 c.c of water to the contents and heat at least one-half hour over an alcohol flame to dissolve the alkali and decompose the sodium peroxide. Next rinse the mixture into a 400–450 c.c. Erlenmeyer flask, by means of hot water, and dilute it to about 250 c.c. Heat the solution nearly to the boiling-point and add concentrated hydrochloric acid

[1] Sometimes the porcelain glaze cracks during heating, in which case the solution should be filtered into the flask.

[2] If the urine is very dilute 50 c.c. may be used.

[3] This moistening of the residue with a small amount of water is very essential and should not be neglected.

slowly until the nickelic oxide, derived from the crucible, is just brought into solution.[1] A few minutes' boiling should now yield a clear solution. In case too little peroxide or too much water was added for the final fusion a clear solution will not be obtained. In this event cool the solution and remove the insoluble matter by filtration.

To the clear solution add 5 c.c. of very dilute alcohol (about 18–20 per cent) and continue the boiling for a few minutes. The alcohol is added to remove the chlorine which was formed when the solution was acidified. Add 10 c.c. of a 10 per cent solution of barium chloride, slowly, drop by drop,[2] to the liquid. Allow the precipitated solution to stand in the cold two days and then filter and continue the manipulation according to the directions given under Total Sulphates, page 770.

Calculation.—Make the calculation according to directions given under Total Sulphates, page 770. Calculate the quantity of sulphur, expressed as SO_3 or S, present in the twenty-four-hour urine specimen.

Interpretation.—See page 771.

(b) Volumetric Procedures

6. Volumetric Determination of Ethereal and Inorganic Sulphates and Total Sulphur.

Fiske's Modification of the Method of Rosenheim and Drummond.[3]

Principle.—The sulphates of the urine are precipitated by means of benzidine solution, the precipitate of benzidine sulphate being filtered off and the sulphuric acid of the compound titrated with N/10 KOH using phenolphthalein as an indicator. This is possible because the benzidine is a very weak base and its sulphate readily dissociates. It is necessary that excess of HCl be avoided in the precipitation process.

Procedure.—(a) Removal of Phosphate.—This step is necessary for the highest accuracy especially where the proportion of phosphorus to sulphur may be high as in half hourly specimens of urine. For 24 hour specimens where the highest accuracy is not desired it may be omitted.

Transfer to a 100 c.c. volumetric flask enough urine (usually 10–20 c.c.) to contain about 10–20 mg. of sulphur as sulphate. Dilute to about 50 c.c. with water. Add one drop of phenolphthalein solution and then concentrated ammonium hydroxide drop by drop to a faint pink color. Add 10 c.c. of 5 per cent ammonium chloride and about 1.5 gm. finely powdered magnesium carbonate. Make to mark, stopper, mix thoroughly by shaking for one minute and let stand for 30 minutes. Filter using a dry filter into a dry flask. This filtrate is used for the three following determinations.

(b) Inorganic Sulphate.—Pipette 5 c.c. of the filtrate into a large Pyrex test tube with flaring lip. Add 2 drops of a 0.04 per cent alcoholic solution of brom-

[1] About 18 c.c. of acid are required for 8 grams of sodium peroxide.

[2] See note (2) at the bottom of p. 770.

[3] Rosenheim and Drummond: *Biochem. J.*, 8, 143, 1914; Fiske: *Jour. Biol. Chem.*, 47, 59, 1921.

phenol blue and 5 c.c. of water. Add approximately N HCl until the last trace of blue disappears and the solution is yellow. Add 2 c.c. of benzidine solution.[1] Let stand for 2 minutes. Add 4 c.c. of 95 per cent acetone and let stand for 10 minutes. Prepare a thin mat of paper pulp in a filtration tube (see Fig. 232).[2] This mat should first be washed with water and then sucked dry. Filter off benzidine sulphate with very gentle suction. Wash down the sides of the test tube with 1 c.c. 95 per cent acetone transferring to filtration tube. Wash twice more with 1 c.c. and finally with 5 c.c. of acetone. Add about 2 c.c. of water and poke the mat with a wire out through the bottom of the tube into the Pyrex test tube, rinsing the wire with a few drops of water. Add 2–4 drops of 0.05 per cent water solution of phenol red. Titrate with 0.02 N NaOH (prepared from 0.1 N NaOH by dilution) the solution being kept hot. At the beginning of the titration the filter tube is kept suspended in the mouth of the test tube and the alkali run through the filter tube to dissolve adherent sulphate. Rinse inner tube with 2–3 c.c. of water, heat solution to boiling so that tube is further washed with condensed steam and finally rinse inner tube with a few c.c. more of water and remove. Titrate to a definite pink color that remains after boiling.

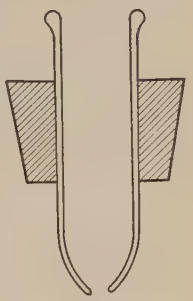


FIG. 232.—FILTRATION TUBE FOR SULPHUR DETERMINATION (*Fiske*).[2]

Calculation.—The sulphate titrates like free sulphuric acid. One c.c. of 0.02 N NaOH is equivalent to 0.32 mg. S. Multiply burette reading by 0.32 to get mg. inorganic S in 5 c.c. of filtrate.

(c) Total Sulphate (Inorganic and Ethereal).—To 5 c.c. of filtrate in a 100 c.c. beaker add 1 c.c. of approximately 3 N HCl. Heat on a water bath to dryness and for ten minutes longer. Transfer to a Pyrex tube with lip using five 2 c.c. portions of water, add 2 c.c. of benzidine solution and proceed as in method for inorganic sulphate. The calculation is the same.

(d) Ethereal Sulphate.—Subtract inorganic from total sulphate. The difference is ethereal sulphate.

(e) Total Sulphur.—Transfer approximately 0.25 c.c. of Benedict's sulphur reagent (20 gm. crystalline copper nitrate and 5 gm. of potassium chlorate in 100 c.c.) and 5 c.c. of filtrate to a small evaporating dish (6 cm.). Evaporate carefully to dryness on wire gauze or hot plate. Increase heat gradually finally igniting at red heat for 2 minutes over free flame. Let cool for 5 minutes. Add 1 c.c. 3 N HCl and evaporate to dryness at low heat. The mixture will turn from green to brown. Transfer to a Pyrex tube with lip with the aid of five 2 c.c. portions of water. Add 1 drop of N HCl and 2 c.c. of benzidine solution. Complete the determination as in the methods above but use in place of the first 1 c.c. portion of acetone in washing 2 c.c. of 50 per cent acetone. The calculation is the same as above.

(f) Neutral Sulphur.—Subtract from the total sulphur the total sulphate as determined above. The difference is neutral or unoxidized sulphur.

[1] Made by shaking 4 gm. benzidine in about 150 c.c. of water in a 250 c.c. flask adding 50 c.c. of approximately N HCl, shaking until dissolved and diluting to mark. Filter if necessary.

[2] Made from glass tubing of 15 mm. diameter shrunken at one end to leave an opening (see Fig. 232) 3 mm. in diameter, cut to a length of 7 cm., and flanged at the cut end. A somewhat elongated tip is desirable.

Interpretation.—The neutral sulphur of the urine is made up of cystine and related bodies, thiocyanate, oxyproteic acids, etc. It makes up ordinarily from 5–25 per cent of the total sulphur of the urine, or on the average 0.2 to 0.4 gram per day calculated as SO_3. The absolute amount is fairly constant for a given individual through wide variations of protein intake, indicating that its origin is mainly endogenous, that is, that it arises principally from the decomposition of tissue protein. On this account the percentage of the total sulphur excretion existing in the neutral form may rise to 25 per cent on a very low protein diet and decrease to 5 per cent on a high protein diet, the absolute amount remaining nearly constant. In fasting percentages as high as 70 have been noted. In many disorders as tuberculosis, cancer, cystinuria, etc., the amount may be relatively and in some cases absolutely increased but no fixed relations have been determined for the various conditions.

Phosphorus

1. Total Phosphates.—Method of Fiske and Subbarow.[1] *Principle.*—Phosphate reacts with ammonium molybdate to form ammonium phosphomolybdate. The latter is reduced by aminonaphtholsulphonic acid to a blue compound which is estimated colorimetrically.

Procedure.—Measure into a 100 c.c. volumetric flask enough urine[2] **to contain between 0.2 and 0.8 mg. of inorganic phosphorus (usually 1 or 2 c.c.). Add water to bring the total volume to 70 c.c., followed by 10 c.c. of 2.5 per cent ammonium molybdate made up in 5 N sulphuric acid (Molybdate I)**[3] **and 4 c.c. of 0.25 per cent aminonaphtholsulphonic acid.**[4] **After the addition of each reagent, the solution should be mixed by gentle shaking.**

[1] Fiske and Subbarow: *J. Biol. Chem.*, 66, 375, 1925. This is a modification of the methods of Bell and Doisy, *J. Biol. Chem.*, 44, 55, 1920 and of Briggs, *J. Biol. Chem.*, 53, 13, 1922 and 59, 255, 1924.

[2] Accurate results can be obtained with proper care using as much as 1.6 mg. of phosphorus. If albumin is present and molybdate gives any sign of turbidity treat the urine with four volumes of 10 per cent trichloroacetic acid, stopper, shake and filter, and repeat the determination using 2 to 10 c.c. of filtrate. If very dilute so that more than 10 c.c. of filtrate must be used follow the method for blood filtrates (see p. 403).

[3] *Molybdate I.*—2.5 per cent ammonium molybdate in 5 N sulphuric acid. Dissolve 25 gm. of the salt in 200 c.c. of water. Rinse into a liter volumetric flask containing 500 c.c. of 10 N sulphuric acid. Dilute to the mark with water and mix.

Standard Phosphate (5 c.c. = 0.4 mg. P).—Dissolve 0.3509 gm. of pure monopotassium phosphate in water. Transfer quantitatively to a liter volumetric flask, add 10 c.c. of 10 N sulphuric acid, dilute to the mark, and mix. The standard keeps indefinitely.

15 *Per Cent Sodium Bisulphite.*—The solution must be free from turbidity before it can be used. Freshly prepared sodium bisulphite solutions may not filter clear, in which case 2 or 3 days standing (before filtering) will be necessary. Keep well stoppered.

20 *Per Cent Sodium Sulphite.*—Because it is likely to be of better quality, it is preferable to use the crystalline sulphite [$Na_2SO_3.7H_2O$]. Dissolve 200 gm. of this in 380 c.c. of water. Remove any suspended matter by filtration, and keep stoppered.

10 *N Sulphuric Acid.*—450 c.c. of concentrated sulphuric acid added to 1300 c.c. of water.

[4] 0.25 Per Cent Aminonaphtholsulphonic Acid—Dissolve 0.5 gm. of the dry powder in 195 c.c. of 15 per cent sodium bisulphite, add 5 c.c. of 20 per cent sodium sulphite, stopper, and shake until dissolved. If the bisulphite solution is old, more than 5 c.c. of sulphite will

At the same time transfer to a similar flask 5 c.c. of the standard phosphate solution (containing 0.4 mg. of phosphorus), 65 c.c. of water, and the same reagents that were added to the urine sample. Dilute the contents of each flask to the mark, mix, and compare in the colorimeter after 5 minutes.

Calculations.—With the standard set at 20 mm., 8 divided by the reading will give the inorganic phosphorus content of the sample in mg. Calculate, in terms of P_2O_5, the total phosphate content of the 24-hour urine specimen.

Interpretation.—The excretion of phosphoric acid is extremely variable but on the average the total output for the 24 hours is about 2.5 grams expressed as P_2O_5. Ordinarily the total output is mainly in the form of phosphates and is distributed between alkaline and earthy phosphates in the ratio of 2:1 but this is likewise inconstant. The greater part of the phosphate excretion arises from the ingested food, either from the preformed phosphates or more especially from the organic combinations as phospho- and nucleoproteins. The excretion is consequently very largely dependent upon the phosphorus content of the diet. Some of the phosphoric acid results from the breakdown of the tissues of the body, and this endogenous phosphoric acid excretion is increased in conditions of increased metabolism as in fevers. The findings in pathological conditions have been somewhat contradictory due to lack of control of diet. The so-called "phosphaturias" nearly always represent decreased acidity and not increased phosphate content of the urine. Such conditions are, however, significant as indicating a possible tendency to the formation of phosphatic calculi.

2. Total Phosphates.—*Uranium Acetate Method.—Principle.*—Standard uranium acetate is run into a measured quantity of urine until all of the phosphate has been precipitated as insoluble uranium phosphate. An excess of uranium is indicated by a reddish coloration with potassium ferrocyanide. This method is accurate and gives practically the total phosphorus of urine inasmuch as the latter exists generally almost entirely as phosphates.

Procedure.—To 50 c.c. of urine in a small beaker or Erlenmeyer flask add 5 c.c. of a special sodium acetate solution[1] and heat the mixture to the boiling-point. From a burette run into the hot mixture, drop by drop, a standard solu-

be needed—in that event add more sulphite 1 c.c. at a time, shaking after each addition, until solution is complete. This reagent can be prepared in a few minutes (the powder need not be very accurately weighed), and if not left exposed to the air it should keep about 2 weeks. The solution is more stable the higher its acidity, hence no more sulphite should be added than is needed to dissolve the reducing agent. For method of preparation of the naphtholsulphonic acid see blood phosphate method p. 403.

[1] The sodium acetate solution is prepared by dissolving 100 grams of sodium acetate in 800 c.c. of distilled water, adding 100 c.c. of 30 per cent acetic acid to the solution, and making the volume of the mixture up to 1 liter with water.

tion of uranium acetate[1] until a precipitate ceases to form and a drop of the mixture when removed by means of a glass rod and brought into contact with a drop of a solution of potassium ferrocyanide[2] on a porcelain test-tablet produces instantaneously a brownish-red coloration. Take the burette reading and calculate the P_2O_5 content of the urine under examination.

Calculation.—Multiply the number of cubic centimeters of uranium acetate solution used by 0.005 to determine the number of grams of P_2O_5 in the 50 c.c. of urine used. To express the result in percentage of P_2O_5 multiply the value just obtained by 2, e.g., if 50 c.c. of urine contained 0.074 gram of P_2O_5 it would be equivalent to 0.148 per cent.

3. **Earthy Phosphates.**—*Principle.*—The earthy phosphates are precipitated by making the urine alkaline. The precipitate is filtered off, dissolved in acid and titrated with uranium acetate.

Procedure.—To 100 c.c. of urine in a beaker add an excess of ammonium hydroxide and allow the mixture to stand 12–24 hours. Under these conditions the phosphoric acid in combination with the alkaline earths, calcium and magnesium, is precipitated as phosphates of these metals. Collect the precipitate on a filter paper and wash it with very dilute ammonium hydroxide. Pierce the paper, and remove the precipitate by means of hot water. Bring the phosphates into solution by adding a small amount of dilute acetic acid to the warm solution. Make the volume up to 50 c.c. with water, add 5 c.c. of sodium acetate solution, and determine the P_2O_5 content of the mixture according to the directions given under the previous method.

Calculation.—Multiply the number of cubic centimeters of uranium acetate solution used by 0.005 to determine the number of grams of P_2O_5 in the 100 c.c. of urine used. Since 100 c.c. of urine was taken this value also expresses the percentage of P_2O_5 present.

Calculate the quantity of earthy phosphates, in terms of P_2O_5, present in the 24-hour urine specimen.

The quantity of phosphoric acid present in combination with the alkali metals may be determined by subtracting the content of earthy phosphates from the total phosphates.

Interpretation.—Ordinarily the earthy phosphates make up from 30–40 per cent of the total phosphate excretion. The amount varies with the excretion of calcium and magnesium (see pp. 784 and 786).

[1] *Uranium Acetate Solution.*—Dissolve about 35.0 grams of uranium acetate in 1 liter of water with the aid of heat and 3–4 c.c. of glacial acetic acid. Let stand a few days and filter. Standardize against a phosphate solution containing 0.005 gram of P_2O_5 per cubic centimeter. For this purpose dissolve 14.721 grams of pure air-dry sodium ammonium phosphate [$NaNH_4HPO_4+4H_2O$] in water to make a liter. To 20 c.c. of this phosphate solution in a 200 c.c. beaker add 30 c.c. of water and 5 c.c. of sodium acetate solution (see above) and titrate with the uranium solution to the correct end reaction as indicated in the method above. If exactly 20 c.c. of uranium solution are required 1 c.c. of the solution is equivalent to 0.005 gram P_2O_5. If stronger than this dilute accordingly and check again by titration.

[2] A 10 per cent solution of potassium ferrocyanide is satisfactory. Cochineal in 30 per cent alcohol may be used as an indicator. If employed it is added directly to the urine after the uranium acetate titration produces no further precipitate. A green color is the end reaction. The use of cochineal is more convenient but rather less accurate than the procedure involving the use of the ferrocyanide.

4. Total Phosphorus.—(*a*) *Neumann's Procedure.—Principle.*—The organic matter is destroyed by digestion with a mixture of sulphuric and nitric acids or some other oxidizing agent. The phosphorus is then precipitated as the phosphomolybdate and determined gravimetrically or volumetrically.

Preparation of the Solution.—Pipette 10 c.c. of urine (or an amount of substance containing about 20 mg. of P_2O_5) into a Kjeldahl flask. Add 10 c.c. of a mixture of equal parts of concentrated H_2SO_4 and concentrated HNO_3. Digest over a low flame until red fumes cease to come off. If the mixture darkens due to the charring action of the sulphuric acid, add nitric acid from a separatory funnel a few drops at a time and continue the digestion. When the mixture remains clear on evaporation to the point where white sulphuric fumes come off the digestion is completed by heating for 10–15 minutes longer. Cool and transfer the solution to a 400 c.c. Erlenmeyer flask with the aid of enough water to make a total volume of about 75 c.c.[1]

Instead of oxidizing the material as described above it may be ignited with magnesia to destroy organic matter. About 2 grams of the solid substance or 25 c.c. of urine (previously evaporated nearly to dryness) are mixed with a little more than an equal bulk of magnesium oxide in a porcelain dish of about 30 c.c. capacity. Five c.c. of magnesium nitrate solution (see Reagents and Solutions, Appendix) are added and the mixture heated very gently at first, then gradually to bright redness. The mixture is cooled and transferred with water to a 250 c.c. flask. An excess (20–30 c.c.) of HCl are added and the mixture boiled a few minutes. Remove from the flame and add at once enough barium chloride solution to precipitate any sulphate present. Cool, make to mark, filter and take an aliquot for analysis.

Precipitation of the Phosphomolybdate.—Neutralize the solution with ammonia, make slightly acid with nitric acid, and add 15 grams of ammonium nitrate in substance (or 25 c.c. of a 60 per cent solution). Heat on a water-bath to 60–65°C. (not higher) and add 30–40 c.c. of molybdate solution,[2] **stir and let stand for about 15 minutes at 60–65°. Filter at once through a small paper,**[3] **washing the precipitate twice by decantation with 1 per cent potassium nitrate solution using about 25 c.c. each time, stirring up the precipitate well in each case, and allowing to settle. Transfer the precipitate to the filter and wash with**

[1] In the case of urine it is possible to neutralize this acid solution with ammonia, make it acid with acetic acid and titrate with uranium acetate as in the preceding method.

[2] Made by adding 5 c.c. of concentrated HNO_3 to 100 c.c. of the ordinary molybdate solution (see Reagents and Solutions, Appendix).

[3] It is better to use a special filter tube of about 1¼ inches diameter (similar to a Gooch filtering tube) in which is placed a perforated porcelain plate 1⅛ inches in diameter, covered with a layer of asbestos ⅛ inch thick. Filtration is carried out with suction and is very rapid. Ordinary Gooch crucibles lined with asbestos may also be used but are not so satisfactory. The asbestos used should be specially prepared (see "Reagents and Solutions" Appendix). For a good discussion of the details of procedure and sources of error of this volumetric method see Hibbard: *J. Ind. Eng. Chem.*, 5, 998, 1913.

1 per cent potassium nitrate solution until two fillings of the filter (collected separately) do not greatly diminish the color produced with phenolphthalein by 1 drop of the standard alkali.

Titration of the Phosphomolybdate.—Transfer the precipitate and filter back to the original beaker and dissolve in a small excess of N/5 NaOH (about 2–3 c.c. more than required to completely dissolve the yellow precipitate). Add about 100 c.c. of boiled and cooled water and a few drops of phenolphthalein as an indicator (a red color should be observed indicating excess of NaOH) and titrate the excess of NaOH with N/10 acid.

Calculation.—Divide the number of cubic centimeters of N/10 acid required by 2 and subtract from the number of cubic centimeters of N/5 NaOH used. This gives the number of cubic centimeters of N/5 NaOH required. Multiply by 0.618 (the equivalent of 1 c.c. of N/5 NaOH in P_2O_5) and obtain the number of milligrams of P_2O_5 in 10 c.c. of the urine analyzed. Calculate the daily output of P_2O_5 in this case from the 24-hour volume.

Interpretation.—Nearly all of the phosphorus of the urine exists as alkali and earthy phosphates. Consequently the total phosphorus varies in the same way as the total phosphates (which see). A small portion of the phosphorus of the urine may exist in organic combination though never in a reduced form. This organically bound phosphate may amount to from 1–4 per cent of the total phosphorus excretion. Little is known with regard to the compounds in which it occurs. Possibly some glycerophosphoric acid may occur either free or as lecithin.

Gravimetric Modification.—The phosphorus may be determined somewhat more accurately by substituting a gravimetric procedure for the above titration. In this case the washed phosphomolybdate precipitate is dissolved on the filter paper with ammonium hydroxide and hot water to make a volume of not more than 100 c.c. Nearly neutralize with HCl, cool, and add about 10 c.c. of magnesia mixture (see Appendix) from a burette. Add slowly (about 1 drop per second) stirring vigorously. After 15 minutes add 12 c.c. of ammonium hydroxide solution (sp. gr. 0.90). Let stand for some time (two hours is usually enough) then filter and wash the precipitate with 2.5 per cent ammonia until practically free from chlorides. Ignite to whiteness or to a grayish-white ash and weigh. Multiply the weight of magnesium pyrophosphate thus obtained by 0.637 to get the weight of P_2O_5.

Calculation.—Calculate as explained above.

Chlorides

1. Volhard-Arnold Method.—*Principle.*—The urine is acidified with nitric acid and the chlorides precipitated with a measured excess of standard silver nitrate solution. The silver chloride formed is filtered off and in the filtrate the excess silver nitrate is titrated back with standard ammonium thiocyanate solution. Ferric ammonium sulphate is used as an indicator. A red color due to the forma-

tion of ferric thiocyanate indicates that an excess of thiocyanate is present and that the end point has been reached.

Procedure.—Place 10 c.c. of urine in a 100 c.c. volumetric flask, add 20–30 drops of nitric acid (sp. gr. 1.2) and 2 c.c. of a cold saturated solution of ferric alum. If necessary, at this point a few drops of 8 per cent solution of potassium permanganate may be added to dissipate the red color. Now slowly run in a known volume of the standard silver nitrate[1] solution (20 c.c. is ordinarily used) in order to precipitate the chlorine and insure the presence of an excess of silver nitrate. The mixture should be continually shaken during the addition of the standard solution. Allow the flask to stand 10 minutes, then fill it to the 100 c.c. graduation with distilled water and thoroughly mix the contents. Now filter the mixture through a dry filter paper, collect 50 c.c. of the filtrate and titrate it with standardized ammonium thiocyanate solution.[2] The first permanent tinge of red-brown indicates the end point. Take the burette reading and compute the weight of sodium chloride in the 10 c.c. of urine used.

Calculation.—The number of cubic centimeters of ammonium thiocyanate solution used indicates the excess of standard silver nitrate solution in the 50 c.c. of filtrate titrated. Multiply this reading by 2, inasmuch as only one-half of the filtrate was employed, and subtract this product from the number of cubic centimeters of silver nitrate (20 c.c.) originally used, in order to obtain the actual number of cubic centimeters of silver nitrate utilized in the precipitation of the chlorides in the 10 c.c. of urine employed.

To obtain the weight in grams of the sodium chloride in the 10 c.c. of urine used, multiply the number of cubic centimeters of the standard silver nitrate solution, actually utilized in the precipitation, by 0.010. If it is desired to express the result in percentage of sodium chloride move the decimal point one place to the right.

In a similar manner the weight, or percentage of chlorine may be computed using the factor 0.006 instead of 0.010.

Calculate the quantity of sodium chloride and chlorine in the 24-hour urine specimen.

Interpretation.—From 10–15 grams of chlorine, expressed as sodium chloride, are excreted per day, on the average, by normal adults. The amount is, however, closely dependent upon the chloride content of the food ingested. In fasting, the chloride excretion falls rapidly to a very minimal quantity. On high water ingestion it is increased.

[1] Standard silver nitrate solution may be prepared by dissolving 29.061 grams of silver nitrate in 1 liter of distilled water. Each cubic centimeter of this solution is equivalent to 0.010 gram of sodium chloride or to 0.006 gram of chlorine.

[2] This solution is made of such a strength that 1 c.c. of it is equal to 1 c.c. of the standard silver nitrate solution used. To prepare the solution dissolve 13 grams of ammonium thiocyanate, NH_4SCN, in a little less than a liter of water. In a small flask place 20 c.c. of the standard silver nitrate solution, 5 c.c. of the ferric alum solution and 4 c.c. of nitric acid (sp. gr. 1.2), add water to make the total volume 100 c.c. and thoroughly mix the contents of the flask. Now run in the ammonium thiocyanate solution from a burette until a permanent red-brown tinge is produced. This is the end-reaction and indicates that the last trace of silver nitrate has been precipitated. Take the burette reading and calculate the amount of water necessary to use in diluting the ammonium thiocyanate in order that 10 c.c. of this solution may be exactly equal to 10 c.c. of the silver nitrate solution. Make this dilution and titrate again to be certain that the solution is of the proper strength.

In pneumonia and certain other acute infectious diseases the excretion of chlorides may be markedly diminished particularly during the periods in which exudates are forming. In convalescence and with resolution of the exudates the chlorine excretion rises again. A decrease has also been noted in nephritis associated with edema.

2. Volhard-Harvey Method.[1]—*Principle.*—This procedure differs from the Volhard-Arnold method in that the excess of silver nitrate is titrated directly without filtering and hence in the presence of the silver chloride. The procedure is thus more rapid but the exact end point is more difficult to determine.

Procedure.—Introduce 5 c.c. of urine into a small porcelain evaporating dish or casserole and dilute with about 20 c.c. of distilled water. Precipitate the chlorides by the addition of 10 c.c. of standard silver nitrate solution[2] and add 2 c.c. of acidified indicator.[3] Now run in a standard ammonium thiocyanate solution[4] from a burette until a faint red-brown tint is visible throughout the mixture. This point may be determined readily by permitting the precipitate to settle somewhat. Calculate the sodium chloride value as indicated below.

(If a red tint is produced when the first drop of thiocyanate is added an additional 10 c.c. of the standard silver nitrate solution must be introduced. The titration should then proceed as above described and proper allowance made in the calculation for the extra volume of silver nitrate employed.)

Calculation.—Since 2 c.c. of the ammonium thiocyanate solution is equivalent to 1 c.c. of the silver nitrate solution, divide the burette reading by 2 and subtract the quotient from 10 c.c., the quantity of silver nitrate solution taken. This value is the number of cubic centimeters of silver nitrate solution actually used in the precipitation of the chlorides. As 1 c.c. of the silver nitrate solution is equivalent to 0.01 gram of sodium chloride, the number of cubic centimeters of silver nitrate solution used multiplied by 0.01 gram will give the weight of sodium chloride in the 5 c.c. portion of urine used. The weight of chlorine may be computed by using the factor 0.006 instead of 0.01.

Calculate the weight of sodium chloride and chlorine in the 24-hour urine specimen.

[1] Harvey: *Archives of Internal Medicine*, 6, 12, 1910.

[2] See p. 781.

[3] This is prepared as follows: To 30 c.c. of distilled water add 70 c.c. of 33 per cent nitric acid (sp. gr. 1.2) and dissolve 100 grams of crystalline ferric ammonium sulphate in this dilute acid solution. Filter and use the filtrate which is a saturated solution of the iron salt. This single reagent takes the place of the nitric acid and ferric alum as used in Volhard-Arnold method, and insures the use of the proper quantity of acid.

[4] This is a solution of ammonium thiocyanate of such a strength that 2 c.c. is equivalent to 1 c.c. of the silver nitrate solution. First make a concentrated solution by dissolving 13 grams in 1 liter of water. To determine the requisite dilution to make such a solution that 2 c.c. shall be equivalent to 1 c.c. of the silver nitrate solution proceed as follows: Introduce 10 c.c. of the silver nitrate solution into a small porcelain evaporating dish or casserole, add 30–50 c.c. of distilled water, 2 c.c. of the acid indicator and titrate as described above with the ammonium thiocyanate solution. The total volume of the concentrated thiocyanate solution excluding that used in this titration is divided by the burette reading and the result multiplied by the difference between this burette reading and 20 c.c. This will give the volume of distilled water which must be added to the concentrated thiocyanate solution to render 2 c.c. equivalent to 1 c.c. of the silver nitrate solution.

A "short cut" method of calculating the 24-hour output of sodium chloride consists in subtracting the burette reading from 20 c.c., multiplying this value by the total urine volume and pointing off three places.

Interpretation.—See page 781.

Total Fixed Base

Method of Fiske.[1] *Principle.*—The fixed bases sodium, potassium, calcium and magnesium are converted into sulphates and the combined sulphuric acid titrated by the benzidine method.

Procedure.—Measure into a large lipped test tube (200 × 20 mm.) a sample of urine containing preferably between 10 and 25 mg. NaCl but not more than 5 mg. of inorganic phosphorus. Add 1 c.c. of approximately 4 N sulphuric acid and 0.5 c.c. concentrated nitric acid and boil down until white fumes appear. If the residue does not soon become colorless after this stage has been reached, cool slightly, add a few more drops of nitric acid, and continue the heating. When the remaining drop of sulphuric acid has become clear and colorless, let cool for a few minutes, and wash into a test tube which is marked at 25 c.c., with four 2 c.c. portions of water. Add a drop of saturated alcoholic solution of methyl red. Neutralize with powdered ammonium carbonate until the color of the indicator just begins to change, and restore the pink color by adding 4 N sulphuric acid 1 drop at a time. Heat to boiling and add more acid to restore the pink color if this is necessary. Add a 10.5 per cent solution of ferric chloride crystals in 0.2 N HCl in the proportion of 0.1 c.c. for each mg. of inorganic phosphorus present, shake and run in 1 c.c. of a 5 per cent solution of ammonium acetate. Add sufficient water to make the total volume 10 or 11 c.c., heat again to boiling and dilute to the 25 c.c. mark with cold water. Insert a rubber stopper and mix by inverting a few times. Filter at once through a dry 9 cm. ashless paper into a dry test tube. Keep the filter nearly filled with liquid as long as possible and collect only 20 c.c. Stopper tube containing filtrate and cool. The phosphate has now been removed.

Transfer 5 c.c. of filtrate to a small platinum dish, add 1 c.c. of approximately 4 N sulphuric acid and evaporate on the water bath until nearly dry. Place the dish on a metal triangle and heat cautiously at first over a microburner gradually raising the flame until fumes have ceased to come off. Let cool, sprinkle over the residue a little powdered ammonium carbonate, and ignite again, finally raising the flame to its maximum and moving the triangle about until each part of the dish has been momentarily subjected to a dull red heat. When the dish has cooled add 2 c.c. of water. Agitate until the residue has dissolved using a rubber-tipped rod to assist in solution if necessary. Transfer the contents of the dish to a large lipped Pyrex test tube rinsing four times with 2 c.c. portions of water. Determine the sulphate according to the benzidine method (see p. 774).

Calculation.—An aliquot of one-fifth of the original urine was used for the determination and 0.02 N NaOH in the titration. Therefore the titration reading is equivalent to the number of c.c. of 0.1 N fixed base in the sample of urine used. Subtract a correction of one per cent for the contraction of the warm solution during the filtration stage.

Interpretation.—The total base excretion combining as it does the sodium, potassium, calcium and magnesium excretions will be influenced by factors effect-

[1] Fiske: *Jour. Biol. Chem.*, 51, 55, 1922.

ing any of these. In acidosis volatile base (ammonia) plays a large part in neutralization but the fixed base excretion is also increased in varying degrees.[1]

Calcium

Determination of Calcium. Method of Shohl and Pedley.[2] *Principle.*—The urine is oxidized with ammonium persulphate. Calcium is precipitated as oxalate and titrated with potassium permanganate. The method is more rapid than the gravimetric.

Procedure.—To 100 c.c. of unfiltered urine in a 250 c.c. Erlenmeyer flask add 5 c.c. of concentrated HNO_3 or H_2SO_4 and one spoonful containing 3.0–4.0 gm. of ammonium persulphate. Insert a funnel in the flask to prevent spattering. Boil and keep near the boiling point on a hot plate or over a low flame for 1 hour or until reduction of the persulphate is complete as evidenced by an absence of frothing when the flask is agitated. The solution at this point is pale green in color. Add 10 c.c. of 2.5 per cent oxalic acid. Cool to room temperature. Neutralize with ammonium hydroxide using one drop of methyl red as an indicator. Cool to room temperature. If the color is now red add a few drops of ammonia to bring to intermediate color between red and yellow (pH 4.8–5.2). Let stand over night. Filter. Whatman No. 50 hardened filter paper 12.5 cm. is satisfactory. Wash precipitate and flask 3 times with distilled water filling the filter two-thirds full each time and allowing to drain. Break a hole in the filter paper and wash back the precipitate into the original flask, first with distilled water and then with hot dilute sulphuric acid bringing the volume to about 100 c.c. Add 10 c.c. of concentrated sulphuric acid and heat to 70–80°C. Titrate with 0.05 N potassium permanganate taking as an end point the first color that persists 15–30 seconds. One c.c. of 0.05 N $KMnO_4$ = 0.001 gm. or 1 mg. of calcium.

Interpretation.—The average urinary excretion of calcium by normal adults lies between 0.1 to 0.4 gram (expressed as CaO) per day. It is dependent very largely upon the amount of calcium in the diet. From 10 to 40 per cent of the ingested calcium ordinarily is excreted by this channel, the greater part being eliminated by the feces. The proportion is dependent particularly on the amount of calcium in the food. If the calcium ingestion is very high the per cent of the total excretion taking place by way of the kidneys will be low, and *vice versa*. As excretion takes place by way of the intestine as well as by the kidneys no conclusions can be drawn from urinary analyses alone. The excretion of calcium may be greatly increased in certain bone disorders as osteomalacia. In others as in rickets the urinary excretion may be very low.

Calcium and Magnesium

McCrudden's Methods.[3]—*Principle.*—Urine contains magnesium, phosphates and a small amount of iron, each of which will interfere

[1] Fiske and Sokhey: *Jour. Biol. Chem.*, 63, 309, 1925.
[2] Shohl and Pedley: *Jour. Biol. Chem.*, 50, 537, 1922.
[3] McCrudden: *Jour. Biol. Chem.*, 7, 83, 1910; 10, 187, 1911.

with the accurate determination of its calcium content if proper conditions of acidity are not maintained during the precipitation. In the following method the proper acidity is attained through the use of sodium acetate and hydrochloric acid, and this with slow addition of the ammonium oxalate reduces the danger of occlusion of magnesium oxalate, calcium phosphate, or ferric phosphate in the calcium oxalate precipitate.

The calcium oxalate precipitate is either ignited and weighed as CaO or determined volumetrically by titration with potassium permanganate. Magnesium is determined in the filtrate from the calcium determination after destruction of the organic matter. It is determined in the usual way by ignition of the magnesium ammonium phosphate precipitate and weighing as the pyrophosphate.

Lyman has suggested a nephelometric method for the determination of calcium in urine and feces.[1]

Procedure for Calcium.—If the urine is alkaline make it neutral or slightly acid and filter. Take 200 c.c. of the filtered urine for analysis. If it is only faintly acid to litmus paper add 10 drops of concentrated hydrochloric acid (sp. gr. 1.20). If the urine is strongly acid it may be made just alkaline with ammonia and then just acid with hydrochloric acid after which the 10 drops of concentrated hydrochloric acid are added. Then add 10 c.c. of 2.5 per cent oxalic acid. Run in slowly with stirring 8 c.c. of 20 per cent sodium acetate. Allow to stand over night at room temperature or shake vigorously for ten minutes. Filter off the precipitate of calcium oxalate on a small paper and wash free from chlorides with 0.5 per cent ammonium oxalate solution. The precipitate may then be dried, ignited to constant weight and weighed as calcium oxide or it may be manipulated volumetrically as described below.

Volumetric Procedure.—If free from uric acid the calcium oxalate precipitate may be washed three times with distilled water, filling the filter about two-thirds full and allowing it to drain completely before adding more. A hole is made in the paper and the calcium oxalate washed into the flask. The volume of the fluid is brought up to about 50 c.c. and 10 c.c. of concentrated sulphuric acid added. Titrate with standard potassium permanganate solution to a pink color which endures for at least a minute.

Calculation.—One c.c. of N/10 permanganate solution is equivalent to 2.8 mg. of CaO. Calculate the daily output of calcium expressed as CaO.

Procedure of Magnesium.—Transfer the filtrate from the determination of calcium as above to a porcelain dish, add about 20 c.c. of concentrated nitric acid and evaporate to dryness. Heat the residue over a free flame until the ammonium salts are destroyed and the residue fuses. After cooling take the residue up with water and a little hydrochloric acid and filter if necessary. Dilute to about 80 c.c., nearly neutralize with ammonia and cool. Add a slight excess of sodium acid phosphate and then ammonia drop by drop with constant stirring until the solution is alkaline and then add enough more slowly with

[1] Lyman: *Jour. Biol. Chem.*, 21, 551, 1915.

constant stirring to make the solution contain one-fourth its bulk of dilute ammonia (sp. gr. 0.96). Allow to stand over night. Filter and wash free from chlorides with alcoholic ammonia solution (1 part alcohol, 1 part dilute ammonia, 3 parts water). The precipitate with filter paper is incinerated slowly and carefully with good supply of air to prevent reduction, in the usual manner, and ignited and weighed as the pyrophosphate.

Calculation.—To obtain the weight of MgO multiply the weight of magnesium pyrophosphate by 0.3624.

Interpretation.—The daily excretion of magnesium by way of the urine usually amounts to between 0.1 and 0.3 gram (expressed as MgO). The amount depends mainly upon the diet. Usually 50 per cent or more of the excreted magnesium is eliminated by the kidneys, the remainder passing out in the feces. The proportion varies, however, and it is impossible to draw any conclusions from the urinary output alone. There may be a retention of magnesium in certain bone disorders accompanying a loss of calcium; in osteomalacia for example. Thus the excretions of calcium and magnesium do not necessarily run parallel.

Determination of Calcium in Ash of Foods or Feces.[1]—Ignite the material in a crucible to a white ash and dissolve the ash with the aid of a little hydrochloric acid. Bring the volume of the ash solution to 75–150 c.c. Make just alkaline with strong ammonia added drop by drop (using litmus paper or alizarin as an indicator). Add concentrated HCl drop by drop until just acid to litmus. Then add 10 drops of concentrated HCl (sp. gr. 1.20), and 10 c.c. of 2.5 per cent oxalic acid. Either of two procedures may then be followed. (*a*) The solution is boiled until the precipitated calcium oxalate is coarsely crystalline, and then an excess of 3 per cent ammonium oxalate is slowly added to the boiling solution and the boiling continued until the precipitate is coarsely crystalline. (If but little calcium is present nothing will precipitate at this point and it is not necessary to add oxalate.) Or (*b*) the flask closed with a rubber stopper is shaken vigorously for ten minutes. An excess of 3 per cent ammonium oxalate is then added. Cool to room temperature. Add 8 c.c. of 20 per cent sodium acetate solution. (In case of ash of feces add 15 c.c.) The solution may either be (*a*) allowed to stand over night or (*b*) stoppered and vigorously shaken for ten minutes. The calcium oxalate is filtered off on a small ash-free paper and washed free from chlorides with 0.5 per cent ammonium oxalate solution. Either of two procedures may next be followed. (*a*) The precipitate and filter are dried, burned in a platinum or porcelain crucible to constant weight as CaO. (*b*) The precipitate is washed three times with cold distilled water, as given under the method for urine and the oxalate titrated with potassium permanganate.

Magnesium is determined in the filtrate from calcium just as given above.

Sodium and Potassium

Determination of Combined Sodium and Potassium.[2]—From 50 to 100 c.c. of urine, depending upon the specific gravity, are oxidized in a Kjeldahl flask with

[1] For another procedure for the determination of calcium in tissues and other biological materials see p. 409. (Corley and Denis: *Jour. Biol. Chem.*, 66, 601, 1925.)

[2] For method for direct quantitative determination of sodium, potassium, calcium, and magnesium in urine and feces see Tisdall and Kramer: *Jour. Biol. Chem.*, 48, 1, 1921.

nitric and sulphuric acids as in the Neumann procedure for total phosphorus (see page 779). To remove the sulphuric acid as completely as possible transfer with the aid of a little water to a platinum dish[3] and evaporate to dryness over a free flame. (The alkalies are in the form of sulphate and do not volatilize.) Dissolve the residue in hot water with the aid of a little dilute hydrochloric acid. Heat to boiling and add barium chloride solution until no more precipitate forms. While still hot add an excess of ammonia and ammonium carbonate. The barium chloride precipitates the sulphates and part of the phosphates: the ammonia in the presence of excess barium precipitates the rest of the phosphates, and the carbonate precipitates the calcium and most of the magnesium, as well as the excess barium. Filter and wash the precipitate well with hot water containing a few drops of ammonia. Evaporate filtrate and washings to dryness and heat the residue to dull redness for a moment. Redissolve in water and treat again with ammonia and ammonium carbonate to remove any remaining alkaline earth metals. Filter and wash as before. Transfer filtrate and washings to a weighed platinum dish, add a few drops of hydrochloric acid and evaporate to dryness. Heat the residue gently to remove ammonium salts and then to dull redness for a moment. Desiccate and weigh. Reheat to constant weight which represents the combined chlorides of sodium and potassium. The reagents used in the determination must be tested and found free from alkali metals or a correction made for the alkali metals present in the reagents used. The sodium is determined by difference after potassium has been estimated by the method given below.

Potassium.—Dissolve the alkali chlorides from the preceding determination in a little water and add a slight excess of 10 per cent platinic chloride over that necessary to precipitate all of the alkali present calculated as sodium chloride (about 17 c.c. being required for each gram of sodium chloride). Evaporate to a syrupy consistency on the water-bath and add about 50 c.c. of 80 per cent alcohol. Stir occasionally for a few minutes. This operation must be carried out in the absence of ammonia vapors. Filter through a weighed Gooch crucible, washing the precipitate with 80 per cent alcohol first thoroughly by decantation and then on the filter, for some time after the filtrate is colorless. Dry at 110–115°C. and weigh.

Calculation.—Multiply the weight of potassium platinic chloride by 0.1941 to obtain the amount of K_2O present. Express as KCl by using instead of this factor the factor 0.30712. Subtract from the weight of total alkali chlorides as determined in the preceding method, the weight of potassium chloride as calculated and obtain the amount of sodium chloride present.

Interpretation.—The average alkali excretion of an adult on a mixed diet is about 2–3 grams of potassium expressed as K_2O and 4–6 grams of sodium expressed as Na_2O. The ratio of Na to K is thus about 5:3. Both the ratio and the absolute amounts of these elements excreted are, however, largely dependent upon the salt content of the diet. Because of the non-ingestion of sodium chloride and the accompanying destruction of potassium-containing body tissues, the urine during fasting contains more potassium than sodium salts. The excretion of the bases, particularly K, may be increased in fevers and in acidosis.

[3] Wilson (*Jour. Biol. Chem.*, 50, 301, 1922) suggests the use of inexpensive *tin* dishes instead of platinum.

Iron[1]

Method of Wolter.[2]—*Principle.*—The urine is ashed, the ash dissolved, and the iron present oxidized to the ferric form by means of hydrogen peroxide. The iron is then determined iodometrically.

Procedure.—The 24-hour specimen of urine is treated with 30 c.c. of concentrated iron-free nitric acid and then evaporated to low volume in a large evaporating dish on the water-bath. Transfer to a small evaporating dish. Heat to dryness on the sand bath and then char, using a small flame. Transfer the charred mass by means of a glass spatula to a crucible. The remaining material in the evaporating dish is transferred with the aid of a little hot water and a rubber "policeman" to a second crucible. Evaporate to dryness on the water-bath and then ash the material in both crucibles. Dissolve the ash in about 30 c.c. of iron-free hydrochloric acid, transfer to an Erlenmeyer flask, add 2 c.c. of hydrogen peroxide and boil for three-quarters of an hour. After cooling, 2 grams of potassium iodide and a few drops of fresh starch paste are added. The liberated iodine is titrated with N/100 thiosulphate solution. Controls should be run on reagents. A correction of 0.32 mg. is usually necessary for the undecomposed hydrogen peroxide. The thiosulphate solution is made up as needed from an N/10 stock solution by dilution. It is standardized against an iron solution containing 2 mg. of iron in 10 c.c. The number of cubic centimeters of thiosulphate used in titration of the iodine set free from the ash solution is multiplied by the iron equivalent of 1 c.c. of the thiosulphate (about 0.2 mg.) to obtain the total amount of iron in the 24-hour specimen of urine. From 1–5 mg. of iron are usually excreted per day.

[1] Another method for the determination of iron is given in Chapter XVIII.
[2] Wolter: *Bioch. Zeit.*, 24, 108, 1910.

CHAPTER XXXII

STUDIES IN METABOLISM[1]

DIETARY DEFICIENCIES; VITAMIN ASSAYS; ULTRAVIOLET IRRADIATION; ETC.

		Page
I.	Manipulatory Procedures	789
II.	Dietary Deficiencies and Vitamins, Vitamin Assays	803
III.	Experiments on Nitrogen Metabolism	849
IV.	Experiments on Carbohydrate Metabolism	863
V.	Experiments on Fat Metabolism	868
VI.	Experiments on Mineral Metabolism	868
VII.	Influence of Water on Metabolism	871
VIII.	Experiments on Acid-base Metabolism	872
IX.	Experiments on Metabolism of Fasting	880

I. MANIPULATORY PROCEDURES

1. Collection and Preservation of the Urine.—In metabolism tests, such as those given in this chapter the accurate collection of the urine for the *exact* 24-hour period is of the *utmost importance.* Proceed as follows: Empty the bladder at a given hour, *e.g.*, 8 a.m. and *discard the urine.* Prepare a *thoroughly clean* bottle of proper size, introduce into it sufficient toluene to cover the bottom of the bottle and use this bottle for the collection of all urine voided from 8 a.m. until 8 a.m. the next day. During the day, when not actually in use for the introduction of a urine fraction, the bottle should be kept in a refrigerator or cold room in order that the sample may not deteriorate before it is examined. Measure the volume of the sample and determine its specific gravity (see Chapter XXVI) and reaction before proceeding to the quantitative estimation of any specific urinary constituents.

[1] Important books on Nutrition and Vitamins include:
Barker, Mosenthal and Hoskins: "Endocrinology and Metabolism," Appleton, 1924.
Benedict, Lusk, Du Bois, Hill, McCollum and Evans: "Lectures on Nutrition" (Mayo Foundation Lectures), Saunders, Philadelphia, 1925.
Berg: "Vitamins: A Critical Survey of the Theory of Accessory Food Factors," Knopf, New York, 1923.
Eddy: "The Vitamine Manual," Williams and Wilkins, Baltimore, 1921.
Ellis and MacLeod: "Vital Factors of Foods, Vitamins and Nutrition," Van Nostrand, 1922.
Funk: "The Vitamines" (translated by Dubin), Williams and Wilkins, 2nd ed., 1922.
Harrow: "Vitamines," Dutton, New York, 1921.
Hess: "Scurvy Past and Present," Lippincott, Philadelphia, 1920.
Lusk: "The Science of Nutrition," Saunders, Philadelphia, 3rd ed., 1917.
McCollum and Simmonds: "Newer Knowledge of Nutrition, third edition, Macmillan, New York, 1925.
Mendel: "Nutrition—The Chemistry of Life," Yale University Press, 1923.
Sherman: "Chemistry of Food and Nutrition," third edition, Macmillan, New York, 1926.
Sherman and Smith: "The Vitamins," Chemical Catalog Co., 1922.

For metabolism work on *dogs*, a cage is used which is provided with a screen bottom. Below this is a tray which slopes toward a central hole through which the urine passes into a bottle containing toluene. Each day's output is collected, filtered to remove hair, etc. and then made up, to a definite volume, usually 500 or 1000 c.c. By this procedure subsequent calculation is greatly facilitated.

To obtain the complete 24-hour secretion of urine of *dogs*, catheterization must be resorted to. Because of the difficulty of catheterizing male dogs, bitches (especially those who have had pups, and hence have stretched vaginas) are used for this type of experimentation. Care must be taken to have the catheter sterile, in order to avoid infection or cystitis. Rubber catheters are sterilized by boiling in water, while metal or glass catheters may be washed and kept in alcohol. The simplest procedure is to use a speculum (a nose dilator is about the right size) to stretch the vagina and then to insert the catheter directly into the urethra, avoiding contact with the vaginal wall. The exact location of the urethral orifice can be determined with a little experience. Force should never be used. After the bladder has been emptied, it is washed out several times with warm water, the washings being collected with the urine and made up to volume as described above. Finally, if it does not interfere with the experiment, it is desirable to introduce about 50 c.c. of warm saturated boric acid solution into the bladder from time to time.

The most satisfactory method for obtaining urine from a *rabbit*, is to hold its head up between one's knees and to apply gentle pressure on the lower abdomen. By this process of "milking," practically all the urine may be obtained.

For the collection of urine in metabolism work on *rats*, Levine and Smith's Metabolism Cage (see Figs. 238 and 239) is employed.

2. Complete Analysis of Urine.—Ingest an ordinary mixed diet (or any special diet) and collect the urine accurately for a 24-hour period (see above). Measure the volume of the sample, determine the specific gravity and preserve the urine (see above) until the following constituents have been determined (for Methods of Analysis, see Chapter XXXI): total solids, titratable acidity, hydrogen ion concentration, total nitrogen, amino-acid nitrogen, ammonia, urea, uric acid, creatinine, total sulphur, ethereal sulphates, inorganic sulphates, neutral sulphur (by difference) total phosphates and sodium chloride.

Calculate the nitrogen and sulphur "partitions," i.e., the percentage of the total nitrogen and sulphur which occur in the different forms and tabulate the data from the complete analysis. Compare your results with those listed in the table on pages 594 and 860.

3. Collection and Preservation of Feces and the Mixing and Weighing for Analysis.— The old methods in vogue in metabolism

work embraced the analysis of *dried* feces. Various investigators later demonstrated that the drying of feces was accompanied by losses and changes of some of the organic constituents of the feces.[1] Therefore the chemical examination of all stools wherever possible should be made on the *fresh* feces. If a study is being made which extends over several days and it is desired to economize time and effort in the chemical examination the daily fecal output or an aliquot portion of each stool may be collected in a friction-top can or pail of suitable size and preserved by thymol and refrigeration.[2] This method has been found satisfactory when the feces are to be examined for inorganic constituents or total nitrogen. For the determination of fat, carbohydrate, etc., the *fresh* stool should be employed.

In the preservation of feces for the determination of total nitrogen the following simple procedure may be used: Introduce each stool into a weighed friction-top can or pail and place the vessel in a cold room or refrigerator.[3] At the end of the period mix the feces thoroughly and analyze weighed portions. In case individual stools are analyzed, the stool should be collected in a *weighed* flat-bottom porcelain dish.[4] After mixing the feces *very thoroughly* the weight of dish, spatula and feces is determined and the weight of the feces secured by difference.[5] A portion of the well-mixed feces is then introduced into a large weighing bottle containing a glass hoe. Desired amounts of feces are then removed for analysis and the exact weight of such amounts obtained by difference.

The daily output of feces in *dogs* is quite variable, so that in metabbolism work it is advisable to collect feces in periods, rather than each day. The stools are collected from the screen bottom and placed in weighed pans. They are dried by adding alcohol, stirring and evaporating on the water bath and weighed. A few drops of sulphuric acid should be added to prevent the loss of ammonia. After drying the feces may be ground in a mill or mortar, to facilitate uniform sampling.

4. "Separation" of Feces.[6]—In order to differentiate the feces which correspond to the food ingested during any given interval it is

[1] Zaitschek: *Pflügers Arch.*, 98, 595, 1903.
Schimidzu: *Bioch. Zeit.*, 28, 237, 1911.
König: *Landw. Vers. Stat.*, 38, 230.
Frear and Holter: *Report, Penn. State College*, p 123, 1891.
Emmett and Grindley: *Jour. Am. Chem. Soc.*, 31, 570, 1909.

[2] Howe, Rutherford and Hawk: *Jour. Amer. Chem. Soc.*, 32, 1683, 1910. This procedure is not satisfactory if fat is to be determined (Smith, Miller and Hawk: *Jour. Biol. Chem.*, 21, 395, 1915). Such feces shows an hydrolysis of fat to fatty acid and a decrease in total fat.

[3] The author uses a brine tank at −12°C. in which the feces are quickly frozen.

[4] The spatula for mixing the feces should be weighed with the dish.

[5] In case it is desired an *aliquot part* of each stool may be placed in a friction-top can or pail and preserved as a "composite sample" for the period.

[6] For other practical work on feces see Chapter XVI.

customary to cause the subject under observation to ingest some substance, at the beginning and end of the period in question, which shall sufficiently differ in color and consistency from the surrounding feces as to render such differentiation comparatively easy. Two "markers" very widely used in such tests are *wood charcoal* and *carmine*. In making an actual separation of feces in a metabolism experiment proceed as follows: Just preceding or in the early part of the first meal (usually breakfast) of the metabolism test, ingest a gelatine capsule (No. 00) containing 0.2-0.3 gram of carmine or charcoal. From this time collect all stools in *flat-bottom* porcelain dishes and examine for the presence of the "marker." All fecal matter containing portions of the marker may be considered as representing the diet in question. This fecal matter should be retained and preserved (see page 790). Just before or in the early part of the first meal (usually breakfast) following the end of the metabolism test a second "marker" in a gelatine capsule should be ingested. The feces should be carefully inspected until the marker makes its appearance. Retain all fecal matter uncolored by the marker, reject the remainder. Frequent difficulties are encountered in the practical separation of feces, but the character of such difficulties will be most satisfactorily impressed by the performance of actual separations.

5. The Use of Agar-agar to Increase Fecal Bulk.—The indigestible hemicellulose agar-agar has the property of absorbing water readily and therefore when ingested it increases the bulk of the feces considerably. This fact is made use of in some forms of constipation and in the determination of metabolic product nitrogen (see Experiment 12, p. 862).

Experiment.—Ingest a uniform diet for four days.[1] Divide the interval into periods of at least two days each,[1] and "separate" the feces by charcoal or carmine (see Exp. 4, above). On the third and fourth days ingest 10 grams of agar-agar at each meal. Collect the feces for each two-day period (see Experiment 4, above), and note the increase in the daily excretion under the influence of the agar ingestion. What was the increase per gram of agar?

6. The Care and Caging of Albino Rats.[2]—The new type of rat cage illustrated in Figs. 233 and 234 is an excellent one for nutrition experiments.[1] Figure 234 shows the individual parts of cage and Fig. 233 the assembled cage.[3] Animals should be kept in a room with a

[1] Longer periods are advisable where great accuracy is desired.

[2] For a more detailed consideration see "The Breeding and Care of the Albino Rat," by Greenman and Duhring, Wistar Institute, Philadelphia, 1923.

[3] Cages and accessories may be obtained from the A. B. Hendryx Company, New Haven, Connecticut. These cages are 9 inches in diameter, 8 inches high, made of ¼ inch mesh galvanized wire netting, and bound at the edge with sheet zinc. The cage has a *double bottom* to prevent access to excreta and is set in an ordinary enamel-ware pan with sides

FIG. 233.—RAT CAGE FOR NUTRITION EXPERIMENTS, ASSEMBLED
As designed by Professor Arthur H. Smith, Yale University.

FIG. 234.—RAT CAGE FOR NUTRITION EXPERIMENTS, SHOWING INDIVIDUAL PARTS.
As designed by Professor Arthur H. Smith, Yale University.

fairly constant temperature (about 70°F.) and not exposed to direct sunlight. Rats with young should be furnished with strips of paper for nesting.[1] Stock animals are fed a mixed diet of ordinary foods or

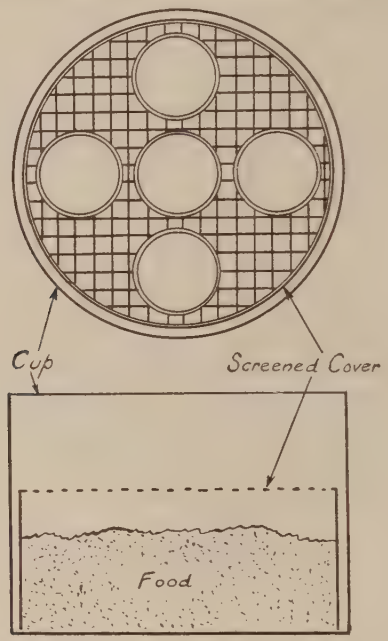


FIG. 235.—FOOD DISH. (*Levine.*)

$2\frac{3}{4}$ inches high and about $9\frac{1}{2}$ inches in diameter at the bottom. Sides of pans should flare enough, so that they may be stocked for storage. Food containers are porcelain cups $1\frac{3}{4}$ inches in diameter and 1 inch high, such as attached to bird cages. In order to prevent the scattering of food a special food dish (see Fig. 235) designed by Dr. Harold Levine, may be employed. Water holders are 4 oz. glass domes fastened to the outside of the cage. A circle of heavy blotting paper should be placed in bottom of pan to absorb urine and water. The paper is covered with a disc of $\frac{1}{8}$ inch mesh wire netting. The hinged top of the cage can be lifted to remove rats or introduce food. Cages should be cleaned at least twice a week and carefully sterilized at least once in two weeks. The cages may be sterilized with steam or washed with cold water and "CN" and dried in the sun. All parts of cages are interchangeable. Bedding is not necessary except for rats with young, if a proper temperature is maintained. Another very useful type of cage has been described by Long.[2] This cage can be taken apart, packed in small space for sterilization and reassembled quickly. Details of construction may be found in the original paper.

[1] Stock animals are better kept in *double bottom* rectangular cages 18 inches long 12 inches wide, and 9 inches high, front and sides of $\frac{1}{2}$ inch netting, reinforced with zinc; back and top of sheet zinc, top being hinged and fastened with a small brass catch in front. The bottom is heavy netting of one inch mesh. The cage rests in a galvanized pan $18\frac{1}{2} \times 12\frac{1}{2} \times 1$ inch. The bottom of the pan is covered with a sheet of heavy blotting paper, held in place by $\frac{1}{8}$ inch mesh wire netting. This netting can easily be removed and cleaned. Flat 4 oz. water bottles may be fastened to the front of the cage by wires. Bedding is not used. A cage is ordinarily used for 4 rats. See Fig. 236.

Just before or after a litter of young is born, the mother and litter may be removed to a special cage, like the others but without bottoms, as very young rats may be crushed against the heavy netting.

The separate sheet of fine netting mentioned above is used. Waste paper strips may be used for bedding. The mother is left with the young rats until they are 3 or 4 weeks old.

The *activity cage* shown in Fig. 237 and the *metabolism cage* shown in Figs. 238 and 239 are very useful for specialized research.

[2] Long: *Anat. Rec.*, 11, 103, 1916.

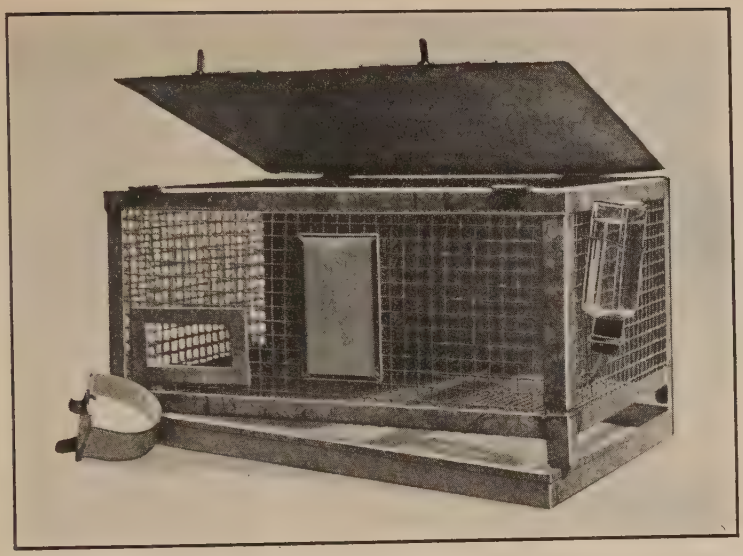


FIG. 236.—RAT CAGE FOR STOCK ANIMALS.

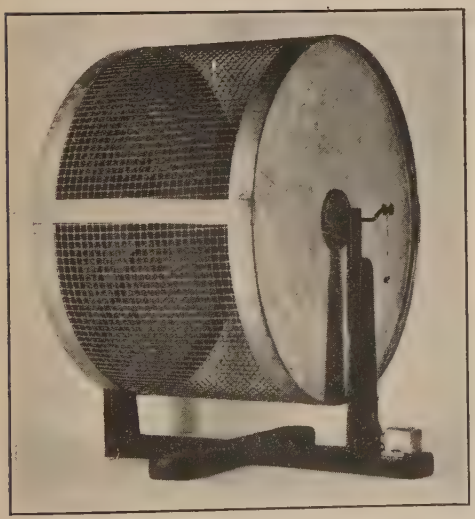


FIG. 237.

FIG. 238.

FIG. 237.—ACTIVITY CAGE.
As used by Drs. Arthur H. Smith and J. E. Anderson, Yale University.

FIG. 238.—METABOLISM CAGE FOR SMALL ANIMALS.
As designed by Dr. Harold Levine, Food Research Laboratories, Inc., New York City, and Professor Arthur H. Smith, Yale university. (*Levine and Smith: Jour. Lab. Clin. Med.*, 11, 168, 1925.)

dog biscuit[1] together with fresh vegetables such as carrots once or twice a week. Nursing mothers and young rats receive also a paste containing whole wheat 66 parts, whole milk powder 33 parts and sodium chloride 1 part. Experimental diets, whenever possible, are made into a semi-solid paste, which the rats cannot easily scatter. Dry food mixtures may be kept for some weeks.

Animals are most conveniently weighed on a spring balance reading from 0–1000 gm. in 5 gm. divisions, weights being estimated to the gram.

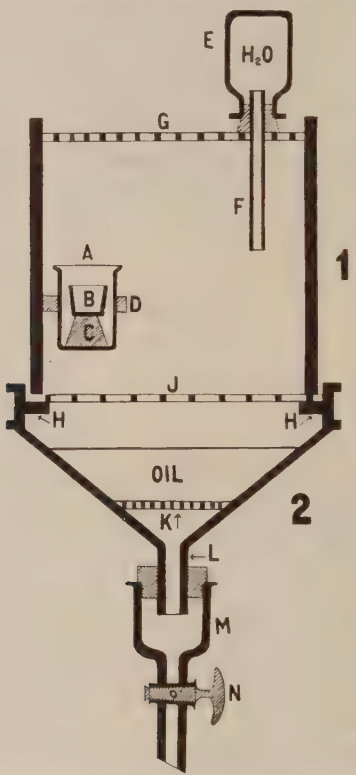


FIG. 239.—CROSS-SECTION OF METABOLISM CAGE FOR SMALL ANIMALS. (See Fig. 238.) (*Levine and Smith.*)

Food cups with food are most readily weighed on a special spring balance.[2] Records are kept of the weekly or semi-weekly food consumption on a form such as illustrated in Fig. 240. Animals from 35–50 gm. in weight are generally used for feeding experiments. They may be marked by clipping the ears with a small shears, punching with an ordinary ticket punch or by staining patches of the fur, and are weighed at least once a week. A form sheet for recording weights and other data

[1] A suitable biscuit may be obtained from the Potter & Wrightington Company, Charles River Avenue, Boston.

[2] The balances may be obtained from the Chatillon Company, New York.

FOOD RESEARCH LABORATORIES, INC.
NEW YORK CITY
FOOD CONSUMPTION
VITAMIN D ASSAY NO.

Date	Rat No. / Diet # / Wt. in Grams: Container plus Food	Food (Grms.)	Scattered in Cage	Food Net	No. of Days	Rat No. / Diet # / Wt. in Grams: Container plus Food	Food (Grms.)	Scattered in Cage	Food Net	No. of Days	Rat No. / Diet # / Wt. in Grams: Container plus Food	Food (Grms.)	Scattered in Cage	Food Net	No. of Days	Rat No. / Diet # / Wt. in Grams: Container plus Food	Food (Grms.)	Scattered in Cage	Food Net	No. of Days

FIG. 240.—FOOD CONSUMPTION CHART.

FOOD RESEARCH LABORATORIES, INC.
NEW YORK CITY
VITAMIN D ASSAY

Client ____ Lab. No. ____ Assay No. ____

Product assayed ____ Prepared by ____

Rats { used as control for this assay, Nos. ____ or, controlled by this assay, Nos. ____

Strain of animals ____ Rearing Diet ____

Check (✓) type of experiment	
Preventive	Curative
Date First X-Ray	

Rat No.	♂		♂		♂		♂		♂		Remarks
Ear Mark											
Litter No.											
Sex											
	Body Weight	Gain or Loss	Body Weight	Gain or Loss	Body Weight	Gain or Loss	Body Weight	Gain or Loss	Body Weight	Gain or Loss	
	Grams		Grams		Grams		Grams		Grams		
Diet	#		#		#		#		#		
Start											
X-RAY SHOWINGS											
1st											
2nd											
3rd											
4th											
5th											
CHEMICAL ANALYSIS OF BONES (Per cent dry matter)											
Calcium (Ca)											
Phosphorus (P)											
POST MORTEM EXAMINATION OF TIBIA											
Macroscopic											
Microscopic											

NOTE: N, normal; +, trace rickets; ++, mild rickets; +++, severe rickets; NH, no healing; H, slight healing; H+, considerable healing; C-, nearly cured; C, cured; D, animal died; K, animal killed intentionally; AD, accidental death; P, paralysis; E, middle ear disease; S, respiratory disorder; i, diarrhea.

FIG. 241.—DATA SHEET FOR VITAMIN ASSAY.

in connection with the experiment (in this case in connection with vitamin D assay, is shown in Fig. 241). Fig. 242 shows a modified type of growth curve chart in which the time coordinates (abscissae) are divided into weeks and days. Provision is made for eighteen experimental weeks. For the usual type of growth curve chart, see Fig. 249.

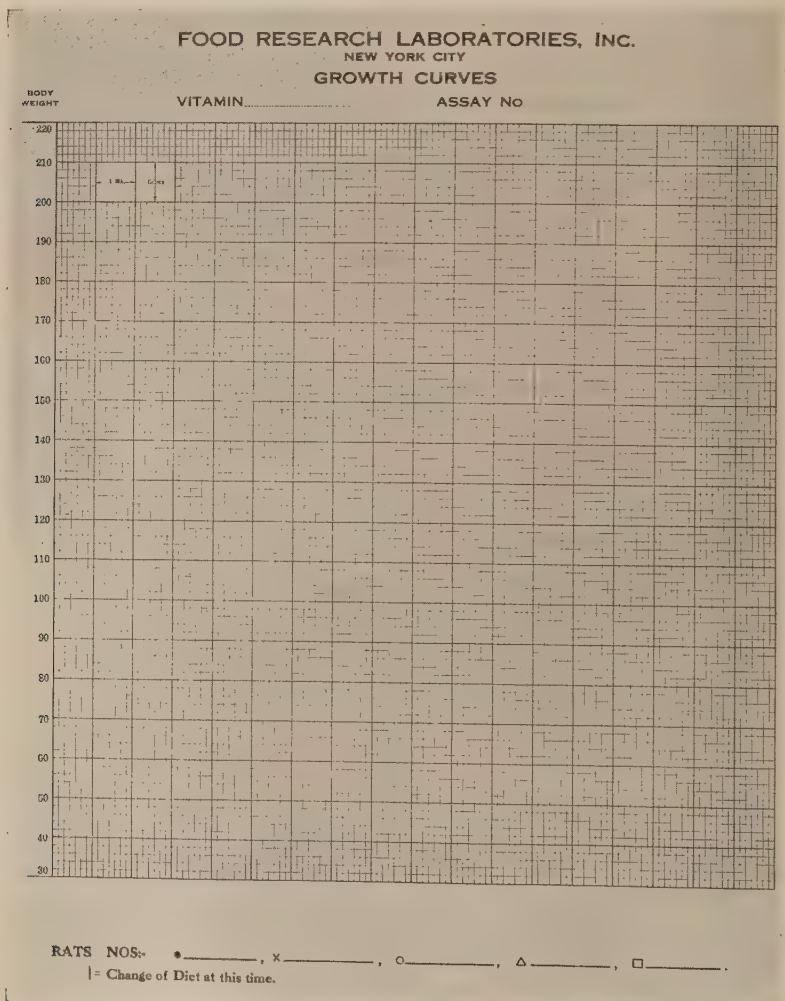


FIG. 242.—GROWTH CURVE CHART.

Inasmuch as vitamin B is present in feces, rats used in assays for this vitamin should be caged in such a manner as to make coprophagy impossible,[1] e.g., in the double bottomed cage shown in Fig. 233, p. 793.

[1] Steenbock, Sell and Nelson: *Jour. Biol. Chem.*, 55, 399, 1923; Dutcher and Francis: *Proc. Soc. Exp. Biol. and Med.*, 21, 189, 1923–24; Cowgill, Deuel and Smith: *Am. J. Physiol.*, 73, 106, 1925; Smith, Cowgill and Croll: *Jour. Biol. Chem.*, 66, 15, 1925.

7. Cowgill's Procedure for Metabolism Experiments on Dogs.[1]—The feeding of a purely "synthetic" food mixture for long periods, in metabolism tests using dogs as experimental animals, is a procedure for the successful accomplishment of which investigators in metabolism have long striven. It has remained for Cowgill to solve this important problem in dietary technique. His work is an extension of that initiated by Karr.[2]

Principle.—The first important principle upon which the new procedure is based is that the diet for the dog shall consist of a mixture of isolated food substances which, when fed, will supply the body with everything that is necessary for proper nutrition, except a single dietary factor. (For the purpose of this discussion we will consider vitamin B as this variable nutritive factor.)

The second important principle is that the variable nutritive factor (in this case vitamin B) shall be administered apart from the mixture of isolated food substances above mentioned.

Procedure.—*The Diet.*[3]—In a test where vitamin B is the variable factor the diet should contain (1) *protein*[4] proper in kind and amount;

[1] Cowgill: *Jour. Biol. Chem.*, 56, 725, 1923. This procedure has been used at Yale University for periods of 5 months and over with excellent results.

[2] Karr: *Jour. Biol. Chem.*, 44, 255, 1920.

[3] *Calculation of the Diet.*—Construct the diet on a kilo of body weight basis, or form what might be called a *kilo unit of food.* Dogs weighing 7 kilos and over require for the maintenance of body weight between 70 and 80 calories per kilo per day. 80 calories has been selected as the number to be desired in the kilo unit of food. This permits *maintenance* in adult animals and allows a slight increase of body weight in growing dogs. Dogs weighing considerably less than 7 kilos require slightly more energy intake in order to maintain their body weight; this fact, however does not invalidate the choice of 80 calories as a unit quantity. Any variation in energy intake from this figure due to the size of the animal will not seriously affect the intake of nitrogen and salt mixture if liberal quantities such as 0.8 gm. of the former and at least 0.2 gm. of the latter be furnished with every 80 calories or kilo unit of food.

"Each kilo unit of food thus prepared furnishes (1) 80 calories, (2) 0.8 gm. of protein nitrogen, (3) from 32 to 36 calories in the form of fat, this foodstuff in grams constituting not over 25 per cent of the whole, (4) 0.4 gm. of agar or of bone ash, (5) 0.2 gm. of salt mixture, and (6) either 7 per cent of butter fat or 2 per cent of cod liver oil as a source of vitamin A. An example of such a diet is given in Table I.

"The reason for feeding so much nitrogen with every 80 calories has been indicated. The amount of nitrogen to be given may very properly be less than this figure; however, if it is less, due consideration should be given to the amino-acid make-up of the protein in order that one or more amino-acids may not thus be made limiting factors in the diet and, therefore, factors influencing the results of the experiments. Should the metabolism of such a condition, *i.e.*, where the low content of an amino-acid is a limiting factor in the diet, be the object of study, one method of approach of course would be to feed a suitable protein on a low plane of nitrogen intake.

Calculation of Kilo Unit of Food.—"First, determine the amount of the protein required to furnish 0.8 gm. or the amount of nitrogen decided upon, using for this purpose the figure for nitrogen content obtained upon analysis of the material. Correct for the non-protein impurities of the sample and calculate the approximate number of calories yielded by the amount of pure protein present in the preparation which furnishes the desired quota of nitrogen. Allot from 32 to 36 calories to fat, divide the particular number selected by 9, and allot the resulting figure in grams *tentatively* to fat. With sucrose supply the remaining calories in an amount sufficient to make a total of 80. Now allot 0.4 gm. to agar (or bone ash) and 0.3 gm. to salt mixtures. Add the several items and thus determine the total weight in grams of the kilo unit of food. Translate all amounts to a percentage basis. If the fat proves to be high—much over 25 per cent—diminish it, making up the calories by an increased amount of sucrose. This final adjustment of fat and sucrose may prove the most laborious of all the steps in the calculation; in diminishing fat and increasing carbohydrate it is necessary to determine a new total weight of the kilo unit and to recalculate the percentages. Finally, divide the fat so that butter fat or cod liver oil form 7 or 2 per cent, respectively, of the entire food mixture."

[4] This would of course not be fed if protein constituted the variable nutritive factor.

TABLE I
KILO UNIT OF CASEIN FOOD[1]

	Amount,	Calories	Percentage	
	gm.			
Casein (12.7 per cent N, 81.9 per cent pure)	6.3	20.8	37.6	
Sucrose	5.84	23.4	34.9	
Lard	2.83	25.5	17.0	24.0
Butter fat	1.17	10.5	7.0	
Bone ash	0.40	.....	2.3	
Salt mixture[2]	0.20	.....	1.2	
Total	16.74	80.2	100.0	

(2) *carbohydrate* and *fat* to furnish satisfactory energy; (3) mineral salts proper in kind and amount; (4) sufficient *vitamin A*;[3] (5) *roughage* and (6) *water*.[4] The vitamin B (the variable factor) should be fed *separate from the rest of the diet*, in a gelatin capsule. Yeast vitamin powder (Harris)[5] is a good source of this vitamin. Cowgill suggests a daily ingestion of 60 mg. per kilo of body weight.

Sources of Dietary Constituents: Protein.—This may be *casein*,[5] *extracted meat*[6] or *commercial coagulated egg albumin*.[7] Casein is to be preferred.

Carbohydrate.—Sucrose is the most desirable source of the carbohydrate. The pure product may be obtained at low cost. Starch or dextrin are not suitable for use in this connection because of the physical characteristics of a diet containing them.

Fat and Vitamin A.—Although cod liver oil is the outstanding source of vitamin A, Cowgill prefers the use of butter fat.[8] Lard may be used as a source of the bulk of the calories.

Mineral Salts.—Cowgill suggests the use of one of the following salt mixtures.

[1] This kilo unit contains 80 calories, 45 per cent of which are furnished by fat, and 0.8 gm. of N.

[2] See Table II.

Calories are calculated on the following basis:
1 gm. of protein yielding 4 calories.
1 gm. of carbohydrate yielding 4 calories.
1 gm. of fat yielding 9 calories.

[3] That the dog is sensitive to the absence of this vitamin has been shown by Steenbock, Nelson and Hart: *Am. Jour. Physiol.*, 58, 14, 1921.

[4] Not mentioned by Cowgill in his procedure. However, a uniform water ingestion is very essential in certain types of metabolism work (see Hawk: *Bioch. Bull*, 3, 420, 1914 and Endocrinology and Metabolism, vol. 3, p. 275, 1924, Appleton).

[5] May be obtained from the Harris Laboratories, Inc., Tuckahoe, New York.

[6] From the Valentine Meat Juice Co., Richmond, Va.

[7] From the Baker's Supply Co., or from any wholesale chemical house.

[8] If ordinary butter with its variable water and salt content is used the diet may become rancid.

TABLE II
SALT MIXTURE (KARR, 1920)[1]

	Gm.
Sodium chloride	10
Calcium lactate	4
Magnesium citrate	4
Ferric citrate	1
Iodine in potassium iodide (Lugol's solution)	a few drops

TABLE III
SALT MIXTURE (COWGILL)[2]

	Per kilo	Percentage
	gm.	
Sodium chloride	0.1050	38.0
Magnesium citrate	0.0905	32.5
KH_2PO_4	0.0336	12.2
$CaHPO_4 \cdot 2H_2O$	0.0215	7.8
Potassium chloride	0.0192	7.0
Ferric citrate	0.0050	1.8
Potassium iodide	0.0013	0.5
Totals	0.2761	99.8

Roughage.—Bone ash has proven very useful for this purpose[3] About 0.4 gram per *kilo unit of food* is the proper amount to feed. If phosphate determinations are contemplated the bone ash should be replaced by agar.

Water.—In certain types of nutrition experiment, the animal should be given a uniform volume of water daily. In such tests as the present where vitamin B is the variable factor, the dog may be permitted water *ad lib* without introducing any error.

Vitamin B.—Theoretically any concentrated from of this vitamin could be used. However, a product which is free from protein and

[1] This salt mixture, when fed along with bone ash on the basis of 0.2 gm. and 0.4 gm., respectively, per kilo unit of food, the latter serving as a source of phosphate, has given successful results with the dog during periods lasting over 5 months.

[2] Where 0.4 gm. of agar-agar per kilo unit is used as a source of roughage, this salt mixture may be used on the basis of 0.3 gm. per kilo unit of food.

Cowgill's salt mixture is supposed to furnish the dog the various essential inorganic substances equal to or slightly in excess of that eliminated through the kidney by a normal organism during 24 hours, these amounts being expressed in terms of grams eliminated per kilo of body weight per day. 0.3 gram of this mixture *per kilo unit* of food contains a liberal amount of the various inorganic substances desired in the synthetic diet. Karr's salt mixture is particularly satisfactory when bone ash is used as roughage. These salt mixtures may be replaced by that of Osborne and Mendel. (*Jour. Biol. Chem.*, 15, 317. 1913) or of McCollum (McCollum and Davis: *Jour. Biol. Chem.*, 23, 231, 1915.)

[3] Gies: *Proc. Am. Physiol. Soc.*, 10, xxii, 1904.

which contains only relatively small amounts of any of the other foodstuffs is the most desirable for metabolism experiments. Such a product should be standardized in order to insure the administration of a sufficient amount of this dietary essential to maintain the appetite. It should also be relatively free from protein and other foodstuffs. The yeast vitamin powder (Harris) has been standardized by Cowgill who found that 60 mg. per kg. body weight was the proper dosage to employ. This powder contains about 8.33 per cent of non-protein nitrogen. Inasmuch as the gelatin capsule used in feeding this yeast powder (No. 00) contains 20 mg. of nitrogen, the animal receives only 3 mg. of nitrogen from gelatin per kg. of body weight per day. A further small increment of 5 mg. of nitrogen per kg. of body weight results from the yeast powder intake.

General Remarks.—It has been claimed that meat is an indispensable article of diet in tests on dogs. As a matter of fact *meat in the feeding of dogs is quite unnecessary.*

Cowgill's work shows that "*monotony of diet* is not *per se* the cause of failure of appetite: loss of the desire to eat is rather an expression either (1) of the failure of the food being fed to nourish the animal properly, or (2) of an adjustment of the dog to its energy requirement when offered more than is necessary of a food mixture that is adequate in all respects.

"When a dog has lost its appetite due to a lack of vitamin B, the desire to eat may be restored within 1 or 2 days by giving to the animal a single large dose of this missing dietary essential: where daily doses, each containing little more than the minimum daily requirement of the vitamin are administered, several days of irregular food intake may elapse before the dog will consume immediately all the food offered. This fact should be considered when interpreting the results obtained by supplying the vitamin to any given animal."

White Mice as Experimental Animals.—The albino rat is the standard animal for nutrition investigations. However, recently the white mouse has been used with success in studies of this character.[1] Some of the advantages of the mouse include a *smaller food requirement, more rapid growth, ease of handling*, and *rapid rate* and regularity of reproduction.

As advantages of the rat over the mouse for nutritional work may be mentioned the fact that the rat has been more extensively studied[2] thus affording ample data for comparison, together with the fact that this animal is hardier than the mouse, not so prone to contract minor

[1] Beard: *Am. Jour. Physiol.*, 75, 645, 658, 668 and 682, 1926.
[2] Donaldson: The Rat, Wistar Institute, Philadelphia, 2nd Ed., 1924.

illnesses, and not so adversely influenced by changes in temperature etc.

In general the albino rat must be considered a more satisfactory experimental animal than the white mouse.

II. DIETARY DEFICIENCIES AND VITAMINS

1. Vitamins.—As a result of the work of different experimenters, six "accessory food substances" or *vitamins* have been shown to be of great importance in nutrition. The exact character of these substances has not been established. There are two distinct growth-promoting vitamins, one soluble in fats and called "Fat-soluble A" or "Vitamin A," the other soluble in water and called "Water soluble B" or "Vitamin B." The third vitamin, "Vitamin C," protects against scurvy and is called the *antiscorbutic* vitamin; the fourth vitamin, "Vitamin D," protects against rickets and is termed the "*antirachitic factor*, while the fifth vitamin, "Vitamin E" is a *reproductive vitamin* or fertility-conferring factor whereas the sixth vitamin is the "P-P factor" or pellagra-preventive factor. The distribution of these 6 vitamins in the various foods eaten by man is given in the table on pages 804 and 805.

When one or more of these essential vitamins is lacking in the diet we have a condition of *avitaminosis*. The term *monavitaminosis* has been suggested to include such conditions as are due to lack of one vitamin and the term *polyavitaminosis* to cover conditions where two or more vitamins are lacking.

The so-called "Deficiency Diseases" include:

1. *Ophthalmia*, or *xerophthalmia*, due to lack of vitamin A.
2. *Beri-beri* due to lack of vitamin B.
3. *Scurvy*, due to lack of vitamin C.
4. *Rickets*, due to lack of vitamin D.
5. *Sterility disease*, due to lack of vitamin E.
6. *Pellagra*, due to lack of "factor P-P."

a. Vitamin A.—This vitamin is essential for growth. Likewise, if it is not present in the diet in adequate amount, an eye disorder termed xerophthalmia or opthalmia results. In the adult a lowered tone of physical fitness accompanies the failure to ingest proper quantities of this important vitamin. Indeed, certain animal tests seem to indicate that an abundance of this factor in the diet tends to promote longevity and a high degree of health and vigor.[1]

[1] Sherman and MacLeod: *Jour. Am. Chem. Soc.*, 47 1658, 1925.

DISTRIBUTION OF THE SIX VITAMINS[1]

Source of the vitamin	Vitamin A	Vitamin B	Vitamin C	Vitamin D[7]	Vitamin E	P-P Factor
	Antiophthalmic and growth-promoting	Anti-neuritic and growth-promoting	Anti-scorbutic	Anti-rachitic and growth-promoting factor	Reproductive or fertility factor	Pellagra-preventive factor
Alfalfa	+++	++	★		+	
Apples	+	++	++	★	★	
Artichoke	+	+				
Asparagus	★	+++	★	★	★	
Bananas	+?	+	++	★	★	
Barley (whole)	+	++	−	★	+[2]	
Beans (kidney, navy or soy)	+	+++	★	★	+[3]	
Beans (string, fresh, raw)	++	++	++	★	★	
Beans (string, cooked)	++	++	+v	★	★	
Beets	★	+	+	★	★	
Bread (white, water)	?	+	−	−	−	
Bread (white, milk)	+	+	?	+	?	
Bread (wholewheat, water)	+	++	?	?	?	
Bread (whole wheat, milk)	++	++	?	+	?	
Butter	+ to +++[4]	−	−	+	+	+[12]
Buttermilk	+	++	+v	?	★	
Cabbage (raw, green)	++	++	+++	−	?	
Cabbage (cooked)	+	++	+v	−	★	
Cabbage (canned)	+	++	?	−	★	
Carrots (raw)	++	+	++	?	★	+
Carrots (cooked)	++	+	+v	?	★	
Cauliflower	+	++	★	★	★	
Celery (bleached stems)	− to +	++	++	−	★	
Celery (green leaves)	++	++	++	−	★	
Cheese (full milk)	++	++	−	★	★	
Cocoanut oil	−	−	−	+	+	
Cod liver oil	+++[5]	−	−	+++[5]	?	?
Corn (yellow)	+	++	−	?	+	
Corn oil (yellow corn)	+v	−	−	?	+	?
Cottonseed oil	?	−	−	?	+	
Cream	+++v	±	+v	★	+ [6]	
Dandelion greens	++	++	+	+	+	
Eggs	+++	+	±	+	+	+
Egg white	−	★	−			
Egg yolk	+++	++	−	+		
Fish (fat)	+	+	★	−		
Fish (lean)	v	+	★	−		
Flour (white)	−	+	−	−		
Flour (whole wheat)	+	++	−	?	+	
Glucose	−	−	−	−	−	
Grapefruit	★	++	++	−		
Hemp seed	+	★	★	★	++	
Honey	±	?	−			
Hydrogenated vegetable oil[9]					+	
Ice Cream	++v	++v	+?			
Lard	− to +	−	−	− to +		
Legumes (germinated)	★	++	++	★	++	
Lemon juice	¬	++	+++	★		
Lettuce	++	++	+++	+	+	+
Margarin (animal fat)	− to +	−	−			
Margarin (vegetable fat)	v[8]	−	−	v[8]		
Meats	− to +	+	?	?	+	++[10]
Milk (fresh, raw)	+++	++	++v	++	±	±[11]
Milk (condensed)	+++	++	+v	+v	±	
Milk (dried, whole)	+++	++	+v	+v	±	
Milk (evaporated)	+++	++	?	+v	±	
Milk (skimmed)	+	+v	+v			
Oats	+	++	?	?	+	
Olive oil	−?	−	−	−	+	

DISTRIBUTION OF THE SIX VITAMINS (*Continued*)

Source of the vitamin	Vitamin A Antiophthalmic and growth-promoting	Vitamin B Antineuritic and growth-promoting	Vitamin C Antiscorbutic	Vitamin D[7] Antirachitic and growth-promoting factor	Vitamin E Reproductive or fertility factor	P-P Factor Pellagra-preventive factor
Onions	− to +	++	++			
Orange juice	±	++	+++[5]		−	
Orange oil	++v					
Parsnips	+	++	★			
Peaches (raw or canned)	++	−?	++			
Peanuts	+	++	★			
Peanut oil	− to +			− to +	− to +	
Peas (fresh)	++	++	+++	±	+ (leaves)	
Pineapple	++	++	+++			
Potato (sweet)	++	++	++	−		
Potato (white, boiled)	+	++	++			
Potato (white, baked)	+	++	+			
Prunes	+v	++	−			
Pumpkin	+					
Radish	−	+	★			
Rice (polished)	−	−	−	−	−	
Rice (whole grain)	+	++	−			
Raspberries	★	★	+++			
Rye (cracked)	+	++	?			
Spinach (raw)	+++	+++	+++	?		+
Spinach (canned)	+++	+	+v	?		
Starch	−	−	−	−	−	
Strawberry juice			++			
Sugar			−	−	−	
Tomato (raw)	++	+++	+++[5]	−		
Tomato (canned)	++	+++	+++[5]	−		
Tomato (cooked)	++	+++	+++	−		
Turnip	− to +	++	+++			
Walnut	★	++	★			
Wheat (whole)	+	++	−	★	+	
Wheat (white flour)	−	+	−	−		
Wheat (grain)	++	+++	+?	?	++	
Wheat (oil of germ)					+++[5]	
Wheat (bran)	+	++	?			
Yeast	−	+++[5]	−	−	−	+++[11]

[1] ± the vitamin is present in minimal amount.
+ the vitamin is present in fair amount.
++ indicates a *good* source of the vitamin.
+++ indicates an *excellent* source of the vitamin.
− no appreciable amount of the vitamin present.
? indicates doubt as to presence or relative amount of the vitamin.
★ evidence lacking or insufficient.
v variable.
[2] Present in sprouted barley.
[3] Present in sprouted beans.
[4] Butter is not a standardized product and its vitamin content varies between wide limits.
[5] The outstanding source of this vitamin.
[6] The higher vitamin content in milk of cows on green pasturage.
[7] Through irradiation by ultraviolet light many of the substances listed in this table may be made to assume antirachitic properties.
[8] The vegetable margarin "Nucoa" has been shown to contain quantities of A and D entirely comparable with the amount present in good butter.
[9] Crisco.
[10] Fresh, lean beef. Preventive dose 200 grams per day.
[11] Preventive dose, 15 grams of Yeast Vitamin Harris Powder or 30 grams of dried yeast.
[12] Not present in all samples of butter.

A relationship between pernicious anemia and vitamin A deficiency has very recently been pointed out.[1] It seems that blood regeneration cannot take place without the presence of vitamin A. A condition similar to human pernicious anemia was produced in albino rats and the rate and intensity of the blood regeneration following the feeding of a "rationally balanced diet" was found to be a function of the quantity of vitamin A present.

Sources of Vitamin A.—The outstanding source of vitamin A is *cod liver oil* (see table p. 804). Other important sources are *butter* (of good quality), *cream, milk, eggs, egg yolk, spinach, carrots, lettuce and*

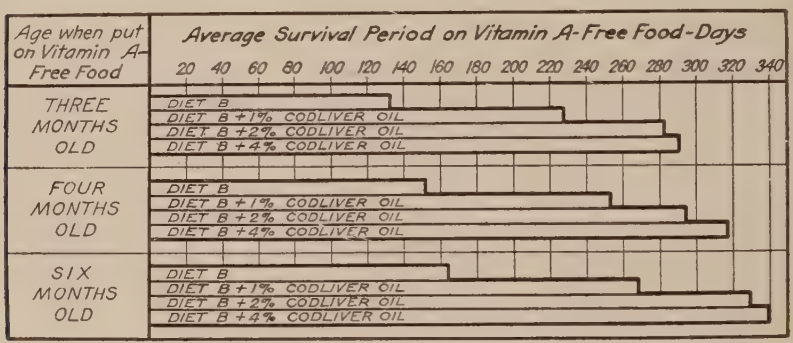

Age when put on Vitamin A-Free Food
Average Survival Period on Vitamin A-Free Food-Days
20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340
THREE MONTHS OLD
DIET B
DIET B +1% CODLIVER OIL
DIET B +2% CODLIVER OIL
DIET B +4% CODLIVER OIL
FOUR MONTHS OLD
DIET B
DIET B +1% CODLIVER OIL
DIET B +2% CODLIVER OIL
DIET B +4% CODLIVER OIL
SIX MONTHS OLD
DIET B
DIET B +1% CODLIVER OIL
DIET B +2% CODLIVER OIL
DIET B +4% CODLIVER OIL

FIG. 243.—EFFECTS OF MODERATE AND OF INCREASINGLY INTENSIVE FEEDING OF VITAMIN A UPON THE STORAGE OF THIS VITAMIN IN THE BODY AT DIFFERENT AGES. (*Sherman and Cammack: Jour. Biol. Chem.*, 68, 69, 1926.)

alfalfa. It has been shown clearly in the case of butter, cream and milk that their vitamin A content depends upon the diet of the cow. Therefore the milk products obtained from cows on green pasturage are higher in vitamin A than these same products produced at other seasons of the year. This is one explanation for the wide variation in the vitamin A content of butter. In recent years, cod liver oil, because of its high vitamin value, has come to occupy an important place not only as a nutritive essential for human beings but as a cattle and poultry food. Bearing in mind that diet regulates the vitamin content of milk, nursing mothers should eat freely of vitamin-rich foods.

Vegetable oils, as a class, are notably deficient in vitamin A. Therefore the margarins made from these oils have a low vitamin value. Only one such margarin (Nucoa) has been proven to have a vitamin A content comparable to that of *good* butter.

It was at one time thought that there was a correlation between *yellow pigmentation* and vitamin A potency. For example *yellow* corn,

[1] Koessler, Maurer and Loughlin: *Jour. Am. Med. Assn.*, 87, 476, 1926. Editorial: *Jour. Am. Med. Assn.*, 87, 672, 1926.

yellow turnips and *yellow* potatoes (sweet) are richer in vitamin A than are the *white* varieties of these food products. However, it was later shown that this relationship was solely a chance one, and that butter as white as lard might have a high vitamin A potency.

Storage of Vitamin A.—When young animals previously fed on a diet rich in vitamin A are deprived of this vitamin they continue to grow for some time due to the vitamin A which has been stored in their body from the previous diet.[1] This is illustrated by Figs. 243 and 244.

A knowledge of the vitamin content of foods is essential in order that one may choose a proper dietary. However, this particular vitamin is of such great importance that it is doubtless a wise practice on the part of children and adults to fortify their vitamin A intake by cod liver oil in some palatable form, *e.g.* an emulsion.

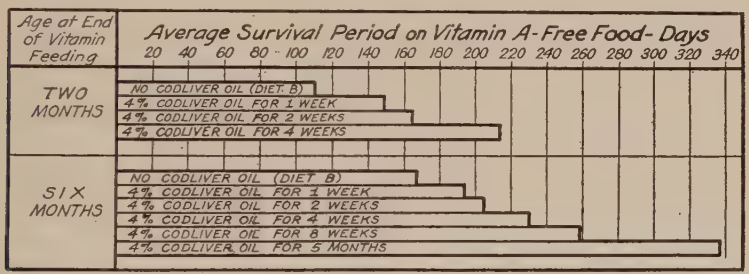

Age at End of Vitamin Feeding
Average Survival Period on Vitamin A-Free Food- Days
20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340
TWO MONTHS
NO CODLIVER OIL (DIET B)
4% CODLIVER OIL FOR 1 WEEK
4% CODLIVER OIL FOR 2 WEEKS
4% CODLIVER OIL FOR 4 WEEKS
SIX MONTHS
NO CODLIVER OIL (DIET B)
4% CODLIVER OIL FOR 1 WEEK
4% CODLIVER OIL FOR 2 WEEKS
4% CODLIVER OIL FOR 4 WEEKS
4% CODLIVER OIL FOR 8 WEEKS
4% CODLIVER OIL FOR 5 MONTHS

FIG. 244.—EFFECTS OF INTENSIVE FEEDING OF VITAMIN A FOR DIFFERENT LENGTHS OF TIME UPON THE BODILY STORE OF THIS VITAMIN AT DIFFERENT AGES. (*Sherman and Cammack: Jour. Biol. Chem.*, 68, 69, 1926.)

Nature and Properties of Vitamin A.—Many attempts have been made to isolate vitamin A in pure form and to definitely establish its identity. The leaders in this work have been Drummond in England and Takahashi in Japan. The latter claims to have secured vitamin A in pure form and to have shown it to be an alcohol, closely related to cholesterol. He calls it "biostearin," gives it the formula $C_{22}H_{44}O_2$ and claims that the presence of 0.0001 per cent. of this substance in the diet maintains the health and growth of the rat.

Vitamin A is relatively stable. It is present, for example, in the yolks of hard-boiled eggs. Likewise cod liver oil may be saponified and the vitamin obtained from the *unsaponifiable* portion of the oil. The cholesterol content of the original cod liver oil is also present along with the vitamin A in the unsaponified material but may be removed by crystallization or precipitation by digitonin.

In the absence of air or oxygen vitamin A may be heated to relatively high temperatures, but when air or oxygen is present the vitamin

[1] Sherman and Boynton: *Jour. Am. Chem. Soc.*, 47, 1646, 1925.
Sherman and Cammack: *Jour. Biol. Chem.*, 68, 69, 1926.

is readily oxidized. Thus the passage of air through cod liver oil for 12 hours at 100° destroys all vitamin A. This "aerated" oil still possesses antirachitic potency (see p. 820). Therefore in the preparation of vitamin A "concentrates" from cod liver oil or other sources it is advisable to carry out the chemical processes, so far as possible, in an atmosphere of carbon dioxide or nitrogen. It is essential also that all distillation procedures be carried out under reduced pressure. Light also exerts a destructive action upon the vitamin. In order to preserve the potency of vitamin A preparations they should be preserved under anaerobic conditions in dark containers. Holmes[1] has demonstrated that cod liver oil should be packed in light-proof containers to prevent loss of vitamin A. While it has not been proven that irradiation by ultraviolet light enhances the antirachitic potency of cod liver oil[2] it has been definitely shown that excessive irradiation destroys vitamin A.[3]

Determination of Vitamin A.[4]—The only way to obtain *accurate* and *quantitative* information as to the amount of vitamin A present in any food or food product is by means of a *biological assay*. The white rat is the classical animal for such assays. Because of the fact that these assays are time-consuming and therefore expensive, attempts have been made to develop colorimetric procedures for the determination of vitamin[5] A (see p. 810). Up to date (September 1926) the efforts to establish a quantitative relationship between the biological assay and the color test have not been successful[6] in the senior author's laboratory or elsewhere. The color reactions, however, serve a useful purpose in yielding *qualitative* information but, in their present state of development, can in no sense be termed accurate, *quantitative* procedures.

Biological Assays for Vitamin A.—Because of the important relationship of this vitamin to rickets, growth and general physical fitness, together with the fact that cod liver oil is the prime source of this vitamin an official method of assaying this oil for vitamin A has been included in the U.S. Pharmacopeia (Vol. X, p. 469, 1925). The directions for this assay follow:

"*Vitamine A Assay for Cod Liver Oil.*—**Cod liver oil may be assayed by the following process. When so assayed the oil should contain at least 50 units per gm. and must be labeled "This unit is not a measure of the antirachitic activity of cod liver oil."**

[1] Holmes and Pigott: *Bost. Med. Surg. Jour.*, 195, 263, 1926.
[2] Wyman, Holmes, Smith, Stockbarger and Pigott: *Bost. Med. Surg. Jour.*, 195, 525, 1926.
[3] Titus *et al.*: *Ind. Eng. Chem.*, 18, 843, 1926.
[4] Sherman and Munsell: *Jour. Am. Chem. Soc.*, 47, 1639, 1925.
Steenbock, Nelson and Black: *Jour. Biol. Chem.*, 62, 275, 1925.
[5] Rosenheim and Drummond: *Bioch. Jour.*, 19, 753, 1925.
Fearon: *Bioch. Jour.*, 19, 888, 1925.
[6] Heller and Guerrant: Private communication from Professor Heller, 1926.
Drummond, Coward and Handy: *Bioch. Jour.*, 19, 1068, 1925.
Carr and Price: *Bioch. Jour.*, 20, 497, 1926.

This assay is based on the minimal amount of cod liver oil necessary to meet specific growth-promoting requirements in a standard test animal. The test animals shall be albino rats from a constant source and bred preferably under the control of the experimenter.

The vitamin A potency of the cod liver oil shall be expressed in units per gram of oil, the unit to be the minimum daily amount required to cure induced symptoms of vitamin A starvation in young albino rats, and to cause a gain in weight of from 10 to 20 gm. within a period of thirty-five days under the conditions of growth and diet specified in this assay.

Place on a vitamin A free diet, rats not less than twenty-five days and not more than twenty-nine days old, and weighing not less than 35 gm., and not more than 45 gm.

The basal diet is to be composed as follows:

Casein, or desiccated fresh meat, rendered free from vitamin A	18%
Salt mixture, such as that of Osborne and Mendel[1] or McCollum and Davis[2]	4%
Starch, sufficient to make	100%

Use sufficient dried brewer's yeast to meet the vitamin B requirements of the animal, mixing it with the basal diet during the vitamin A free period.

The rats shall begin receiving the cod liver oil to be tested after not less than seven days of stationary or declining weight, and from the time of feeding the test oil, they shall be kept in separate cages.

The test period shall continue for thirty-five days and the potency of the oil shall be judged from the rat or rats showing a gain on the thirty-fifth day of between 10 and 20 gm. over the weight at the beginning of the test, and the cure of the induced symptoms of vitamin A starvation.

Each test shall be controlled by at least two standard rats which have received no cod liver oil. These animals may serve as controls for any number of concurrent tests on which the same basal diet is used.

No oil shall be labeled as assayed by the U.S.P. method unless it contains at least 50 units per gm. of oil.

The diet for rats used as breeders shall be composed as follows:

Finely ground whole wheat	66%
Whole milk powder	33%
Sodium chloride	1%

The antirachitic requirements of the breeders shall be assured."

The above assay is in general, as a very logical and worthwhile procedure. It has, nevertheless certain outstanding defects.[3] It will be observed, for example, that each of the dietary constituents is a *powder*. Therefore the collection of *quantitative* data regarding food consumption is most certain to be beset with difficulties due to scattering of food by the animal unless a special food dish is employed (see p.

[1] *Jour. Biol. Chem.*, 15, 317, 1913 (see p. 831).
[2] *Jour. Biol. Chem.*, 33, 55, 1918. (No. 185, Salt mixture.)
[3] Hawk: *Archives of Therapeutics*, March, 1926.
Willimott and Wokes: *Pharm. J.*, 115, 718, 1925.

794). It would seemingly have been far better to have incorporated in the diet fat (free from vitamin A) sufficient to form a *paste* and thus facilitate the feeding process. Permission to use either of two widely different salt mixtures is another defect in the U.S.P. assay. Furthermore experiments carried out in the senior author's laboratory have demonstrated clearly that for certain strains of albino rats the U.S.P. assay procedure should include ultraviolet irradiation.[1]

Color Reactions for Vitamin A.—(1) *The Rosenheim-Drummond Reactions.*[2]

a. Dissolve one to two drops of cod liver oil in about 5 c.c. of an anhydrous fat solvent. Add one drop of concentrated sulphuric acid and shake gently—a deep violet color is a positive test. The color soon fades, or is replaced by a red or brown shade. The violet color is the essential part of the test.

The reaction with sulphuric acid has been known for a long time, and before the advent of vitamins there was a tendency to regard the blue color as being due to impurities in the cold liver oil.[3]

However, Drummond[4] feels quite certain that the blue color produced by action of sulphuric acid on cod liver oil is caused by vitamin A. If this is so, Lewkowitsch's statement that the modern processed medicinal cod liver oils give a fainter blue color than the oils produced by the older rendering and refining methods should give cause for alarm. For it would mean that the present day cod liver oils are suffering a loss of vitamin A content through destructive manufacturing processes.

Rosenheim and Drummond[5] have found that not only does concentrated sulphuric acid produce the deep blue color with cod liver oil but so do the following reagents: arsenic trichloride, dimethyl sulphate, trichloracetic acid and acetyl chloride (the last two only in the presence of zinc chloride). The blue colors produced in these reactions are more or less transient in character. Rosenheim and Drummond found that the arsenic trichloride test possesses the advantage over the other tests of giving a blue color that persists sufficiently long to allow a colorimetric comparison with a suitable color standard.

b. The test, as provisionally formulated, is carried out by adding 1 c.c. of pure arsenic trichloride[6] to one drop of cod liver oil in a test tube and shaking at once. The oil dissolves immediately to form a clear blue solution which in the course of a few seconds assumes a purple tint and gradually fades. The color is characterized by a well defined absorption band extending from λ 550–590. Under the

[1] Unpublished data.

[2] Rosenheim and Drummond: *Biochem. Jour.* 19, 753, 1925.

[3] Lewkowitsch: "Chemistry, Technology and Analysis of Oils, Fats and Waxes," 6th ed., Vol. 2, p. 448, 1922.

[4] Drummond and Watson, *Analyst*, 47, 314, 1922.

[5] Rosenheim and Drummond: *Biochem. Jour.*, 19, 753, 1925.

[6] Carr and Price (*Bioch. Jour.*, 20, 497, 1926) have suggested a reaction based on the use of antimony trichloride. They failed to establish a quantitative relationship between color test and biological assay.

above conditions the band persists for about 5 minutes. The limit of sensitivity is claimed to be 0.05 mg. oil. The reaction is 20 times more sensitive than the sulphuric acid test. The authors make use of a mixture of crystal violet and methylene blue solutions as a colorimetric standard in matching the colors produced in the arsenic chloride reaction.[1]

(2) *Fearon's Color Tests.*—Fearon[2] found that cod liver oil reacts with phosphorus pentoxide to give a blue color, similar to that in the sulphuric acid, and other tests. Although the intensity of the blue color varies in a general way with the vatamin A potency of the oil, the test is nevertheless purely a qualitative procedure.

The test is carried out as follows: Add about 1 gram of phosphorus pentoxide to 5 c.c. of oil (undiluted or dissolved in dry light petroleum) in a dry test tube. Shake gently and a purple color appears. An alternative method is to let one drop of oil fall on the center of a small heap of phosphorus pentoxide on a filter paper. The purple color appears in about one minute and persists until the pentoxide begins to deliquesce. Negative oils give only a yellow color.

Fearon also states that pyrogallol, phloroglucinol, A-naphthol and substituted phenols (vanillin and amidol) all give definite color reactions with cod liver oil in the presence of trichloroacetic acid, and that these color reactions run parallel with the vitamine A potency of the oil.

b. Vitamin B.—This was the first vitamin to be discovered, and like vitamin A is essential for growth. It is also called an *antineuritic vitamin* because polyneuritis, such as that occurring in the disease known as beri-beri, follows a lack of vitamin B in the diet. It has been clearly shown that vitamin B may be fractionated into two distinct entities, one possessing the power to prevent or cure polyneuritis, the other exhibiting growth-promoting potency. The P-P factor of Goldberger[3] (see p. 815) may be identical with the growth-promoting fraction of vitamin B or with bios. Pigeons are generally employed in studying the antineuritic properties of B, and white rats in the investigation of its growth-promoting properties.

[1] In a later communication by Drummond, Coward and Handy (*Biochem. Jour.*, 19, 1068, 1925) the authors state that the quantitative comparison of color tests with rat feeding tests has proven a harder task than they expected, and they do not wish to commit themselves until further work has been completed.

In an unpublished paper by Heller and Guerrant entitled "A Comparison of Potencies of Cod Liver Oils by the Biological and Colorimetric Methods" the technique of the Rosenheim-Drummond test was modified adding ½ c.c. of arsenic chloride to one drop of the oil placed in a depression of a spot plate. The authors state that the color with the arsenic chloride is so unstable that they could not retain it long enough to compare it with a standard in order to give it a set rating by colorimetric methods. Also they state that the use of the test in comparing the color produced in an oil like that of the cod, to the color produced in dissimilar substances is questionable, due to the time required for the color to develop as well as to obscuring colors of the basic substances themselves. (Private communication from Professor V. G. Heller, 1926.)

[2] Fearon: *Biochem. J.*, 19, 888, 1925.

[3] Goldberger: *Medicine*, 5, 79, 1926.

Sources of Vitamin B.—This is the most widely distributed of the vitamins; all natural foodstuffs contain it. (See table p. 804.) Yeast is its leading source and of man's ordinary foods the following contain it in high concentration:[1] *Asparagus*, *beans*, *lettuce*, *prunes*, *spinach*, *egg yolk*, *tomato*, *cabbage*, *turnip*, *milk*, *potato*, etc. The *whole grains* of the cereals have a comparatively abundant supply of vitamin B. Inasmuch as rice polishings and wheat embryo are particularly rich in this vitamin it has been assumed that it resides chiefly in the embryo and to a less extent in the outer layers of the cereal grains. The fallacy of this assumption was first shown by Osborne and Mendel[2] when they demonstrated that the vitamin B of the *wheat* kernel was distributed throughout the entire grain. Bell and Mendel[3] confirmed this finding. In the case of yellow corn, Croll and Mendel[4] report that "practically all of the vitamin B of the whole grain is found in the embryo. The endosperm contains practically no vitamin B, while the bran includes an extremely small amount, if any, of this factor." It is evident therefore that general statements regarding the similar distribution of vitamin B in the kernels of all the cereals are not justified.

Nature and Properties of Vitamin B.—This vitamin is more stable than vitamins A and C. At high temperatures it is more stable in acid than in alkaline solution. The temperatures and other conditions met with in ordinary cooking or canning methods do not destroy this vitamin to any pronounced degree.

Various attempts have been made to isolate vitamin B and determine its chemical nature. Among the most active in this field of research are Seidell[5] and Levene.[6] The latter reports a preparation potent in daily doses of 0.00008 gram. Seidell reports the isolation of an antineuritic base from brewer's yeast which has the formula $C_8H_{15}O_3N_3$, and a molecular weight of 201. Up to date (September, 1926) its exact chemical nature has not been determined.

Vitamin B stimulates the appetite. This has frequently been noted in the author's laboratory. Karr,[7] working on dogs, has shown that the ingestion of a diet deficient in vitamin B leads to loss of appetite, lower food intake and definite polyneuritic symptoms. The animals quickly recover on adding vitamin B to the diet. Cowgill[8] working along similar lines has shown a relationship between vitamin B

[1] Mendel: Nutrition: The Chemistry of Life, Yale University Press, New Haven, 1923.
[2] Osborne and Mendel: *Jour. Biol. Chem.*, 37, 557, 1919.
[3] Bell and Mendel: *Am. Jour. Physiol.*, 62, 145, 1922.
[4] Croll and Mendel: *Am. Jour. Physiol.*, 74, 674, 1925.
[5] Seidell: *Science*, 62, 1597, 1925.
[6] Levene and van der Hoeven: *Jour. Biol. Chem.*, 65, 483, 1925.
[7] Karr: *Jour. Biol. Chem.*, 54, 277, 1920; *ibid.*, 55, 289, 1921.
[8] Cowgill: *Am. Jour. Physiol.*, 57, 420, 1921.

and the appetite of the dog, whereas Osborne and Mendel[1] have shown that there is a minimum daily requirement of vitamin B below which appetite decreases and growth is subnormal.

Cowgill[2] sounds this word of caution: "Various pathological conditions other than vitamin B deficiency may bring about a loss of appetite: in such cases it does not seem reasonable to suppose that the administration of this vitamin will restore the desire to eat; whether it will do so, however, can only be determined by definite experiments planned to answer this question."

In a continuation of these investigations Cowgill and associates[3] have shown "1, that gastric atony is part of the syndrome of an advanced stage of vitamin B deficiency: 2, that successful vitamin therapy applied to such a case is associated with a rapid improvement in tone of the stomach musculature: 3, that the continued feeding of liberal amounts of vitamin B—enough to satisfy completely the body's requirement—to an organism subsisting on a diet adequate in other respects aids in maintaining a satisfactory gastric tonicity."

An interesting relationship between muscular fatigue and vitamin B has been shown by Nelson and associates.[4] They state:

"Fatigue curves produced by animals on diets lacking vitamin B show progressive diminution in amplitude and endurance as the length of time on the diet increases. These curves also show progressive loss of tonicity and an irregularity of contracture that seem very characteristic."

A *quantitative* relation between vitamin B and appetite has been pointed out.[5] It has also been shown that vitamin B does not owe its restorative effect on the appetite to a stimulated gastric secretion, since meat extract, which does not contain vitamin B, and has no effect on the appetite of dogs suffering from vitamin B deficiency, does nevertheless promote the flow of gastric juice.[5]

Data obtained from three widely different species of animals—mouse, rat and dog—indicate that the vitamin B requirement is closely related to metabolism.[6]

In the adult animal the vitamin B requirement is very closely proportional to the product of: (1) the two-thirds power of the weight which may indicate surface or "active protoplasmic mass" or whatever the metabolism depends on, (2) the quantity of energy handled as

[1] Osborne and Mendel: *Jour. Biol. Chem.*, 54, 739, 1922.
[2] Cowgill: *Jour. Biol. Chem.*, 56, 725, 1923.
[3] Cowgill, Deuel, Plummer and Messer: *Am. Jour. Physiol.*, 77, 389, 1926.
[4] Nelson, Baldwin, Riggs and Cunningham: *Am. Jour. Physiol.*, 72, 69, 1925.
[5] Cowgill, Deuel and Smith: *Am. Jour. Physiol.*, 73, 106, 1925.
[6] Cowgill, Smith and Beard: *Jour. Biol. Chem.*, 63, xxiii 1925.

indicated by the total calories utilized from the food, and (3) the body weight. The following formula has been constructed embracing these factors. By it a number called provisionally the "vitamin B constant" may be calculated.

$$\frac{\text{vitamin B}_{per\ day}}{\text{weight}^{\frac{3}{4}}} = \text{K}_{vitamin} \times \text{calories}_{per\ day} \times \text{weight} \qquad (a)$$

whence

$$\text{K}_{vitamin} = \frac{\text{vitamin}_{per\ day}}{\text{calories}_{per\ day} \cdot \text{weight}^{\frac{3}{4}} \cdot \text{weight}} \qquad (b)$$

$$\text{K}_{vitamin} = \frac{\text{vitamin}}{\text{calories} \cdot \text{weight}^{\frac{3}{4}}} \qquad (c)$$

This formula gives good agreement with experimental data for adult rats, dogs and mice. During growth some other factor, as yet undefined, appears to affect the vitamin requirement, $\text{K}_{vitamin}$ during this period having a higher value.

In spite of the fact that the ingestion of vitamin B in sufficient amount does stimulate the appetite and lead to increased physical efficiency there is, so far as the author is aware, no experimental evidence showing how yeast and other vitamin B foods of high potency bring about these alterations in biological response. Thus Cowgill and Mendel[1] have shown in tests on dogs, that "There is no relation between vitamin B and the secretory function of the pancreas, liver and salivary glands." Deuel and Weiss[2] have demonstrated that the basal metabolism (see Chapter XXI) of dogs is not lowered when the animals are deprived of vitamin B, whereas Mitchell and Carman[3] have shown that a ten-fold increase in the vitamin B intake of rats, above their estimated requirement, did not appreciably modify the basal heat production of these animals.

Determination of Vitamin B.—The only way products containing vitamin B can be accurately standardized for this vitamin, is through the biological assay. (See p. 831.) In such work albino rats may be used in the determination of the growth-promoting power of the product and pigeons in the estimation of the antineuritic value.

A color reaction has been suggested for use in the qualitative detection of vitamin B.[4] Later work has shown that the reaction is not specific for this vitamin.[5]

[1] Cowgill and Mendel: *Am. Jour. Physiol.*, 58, 131, 1921.
[2] Deuel and Weiss: *Proc. Soc. Expt. Biol. Med.*, 21, 456, 1924.
[3] Mitchell and Carman: *Am. Jour. Physiol.*, 76, 385, 1926.
[4] Jendrassik: *Jour. Biol. Chem.*, 57, 129, 1923.
[5] Levine: *Jour. Biol. Chem.*, 62, 157, 1924; *ibid.*, 64, 591, 1925. Bezssonov: *Jour. Biol. Chem.*, 64, 589, 1925.

Bios.—This name was given by Wildiers[1] to a "special nutritive principle" necessary for the growth or proliferation of yeast. Williams[2] observed that those substances which were richest in vitamin B were also most potent in stimulating yeast growth and concluded that vitamin B was the stimulating substance. With this conception in mind he then elaborated a method for the quantitative determination of vitamin B. However, Souza and McCollum[3] showed by experiments that the test was not one for vitamin B but for some other substance which influences yeast growth. Therefore "bios" and vitamin B are not identical.

Bios has been obtained in crystalline form by Eddy, Kerr and Williams.[4] They give the tentative formula, $C_5H_{11}NO_3$ to this crystalline substance and show that 0.000005 gr. of the material increases the growth of yeast over twenty-fold. The name "Bios 223" has been given the preparation, the 223 being indicative of its melting point.[5]

Attempts have been made to show that Goldberger's "P-P factor" is identical with bios (see below).

d. The "P-P Factor."—This is the name given to the *pellagra-preventive* substance by Goldberger and associates.[6] This factor, which will cure or prevent pellagra, is present in *lean meat*, *milk*, *vegetables* (especially the green, leafy ones), *eggs*, *yeast*, *carrots*[7] and certain samples of *butter*.[6]

Pellagra is, therefore, classed as a *deficiency disease* and the "P-P factor" apparently must be accorded a place in the vitamin family. On this point Goldberger says "Whether factor P-P is, as at present (Feb. 1926) seems most probable, identical with the so-called growth promoting essential heretofore included (with the antineuritic) in the term 'water-soluble vitamin B,' or whether these are distinct, further investigation must determine."[6]

The disease, pellagra, is more prevalent in the southern part of the United States than elsewhere in this country. In 1917–1918 there were 200,000 cases of the disease in this country, mostly in the southern states.

Pellagrins show the following symptoms: sore tongue, sore mouth, disturbed digestion, diarrhea, and a thickening and bronzing of the skin on hands, forearms, face, neck and other exposed parts of the body,

[1] Wildiers: *La Cellulé*, 18, 313, 1901.
[2] Williams: *Jour. Biol. Chem.*, 29, 495, 1917; 38, 465, 1919; 42, 259, 1920; 46, 113, 1921
[3] Souza and McCollum: *Jour. Biol. Chem.*, 44, 113, 1920.
[4] Eddy, Kerr and Williams: *Jour. Am. Chem. Soc.*, 46, 2846, 1924.
[5] Kerr, Eddy and Williams: *Proc. Soc. Exp. Biol. Med.*, 23, 416, 1926.
[6] Goldberger, Wheeler, Lillie and Rogers: *Public Health Reports*, 41, 297, 1926. Goldberger and Lillie: *ibid.*, 41, 1025, 1926.
[7] Underhill and Mendel: *Public Health Reports*, 40, 1087, 1925.

e.g., the feet and lower limbs if the pellagrin goes bare-foot. The disease is not contagious and is not inherited, and may be contracted at any age. Severe pellagra may be accompanied by mental disturbances. In some of his most recent statements Goldberger calls particular attention to the pellagra-preventing potency of yeast. This is shown by the fact that a daily ingestion of a half ounce of yeast will prevent the disease whereas it requires 7 ounces of fresh lean meat to furnish adequate protection.[1]

Chittenden and Underhill[2] have described a pellagra-like syndrome in dogs which is believed to be identical with "black tongue" which occurs spontaneously in dogs in pellagrous sections.[3] On the basis of further research the conclusion has been reached "that black tongue of dogs is the analogue of pellagra in man and, thus, that the factor P-P is the factor concerned in the prevention and causation of both pellagra and black tongue."[1]

A pellagra-like condition has also been described in the albino rat. One of the chief features in this case is a bilaterally symmetrical dermatitis.[1]

A very comprehensive review of pellagra by Goldberger, may be found in *Medicine*, 5, 79, 1926.

e. Vitamin C.—This was the third vitamin to be discovered and is known as the *antiscorbutic* vitamin because its inclusion in the diet in proper quantity prevents scurvy (scorbutus) or cures this disease if already established.[4]

Albino rats cannot be used as experimental animals in the study of scurvy: guinea pigs are the proper animals to employ. The rat is immune from scurvy because it has the ability to *synthesize* vitamin C. The scurvy symptoms in guinea pigs include: tenderness of joints, developing ordinarily in wrists, ankles and knees in this order, followed in 2–3 days by swelling of joints or spontaneous fracture (in younger pigs). In older pigs the hind legs may stiffen or become paralyzed. This results in the animal assuming the so-called "scurvy position" (See Fig. 245, p. 817.) Hemorrhage of gums may occur and teeth may loosen. The so-called "face-ache position" (see Fig. 246, p. 817) is also characteristic of scurvy. In advanced scurvy there is loss of appetite and body weight.

Sources of Vitamin C.—Fresh fruits and fresh vegetables are the outstanding sources of vitamin C. *Orange juice* and the *tomato* (raw,

[1] Goldberger, Wheeler, Lillie and Rogers: *Public Health Reports*, 41, 297, 1926. Goldberger and Lillie: *ibid*, 41, 1025, 1926.
[2] Chittenden and Underhill: *Am. Jour. Physiol.*, 44, 13, 1917.
[3] Goldberger, *et al.*: *Public Health Reports*, 37, 1063, 1922; 38, 2711, 1923.
[4] Hess: Scurvy—Past and Present, Lippincott, Philadelphia 1920.

canned or cooked) are very rich in this vitamin. Other foods which possess a high antiscorbutic value (see table, p. 804) are *cabbage, lemon juice, lettuce, peas, pineapple, raspberries, spinach* (raw), and *turnip*. Milk is man's best food for general purposes, but it is not the best source

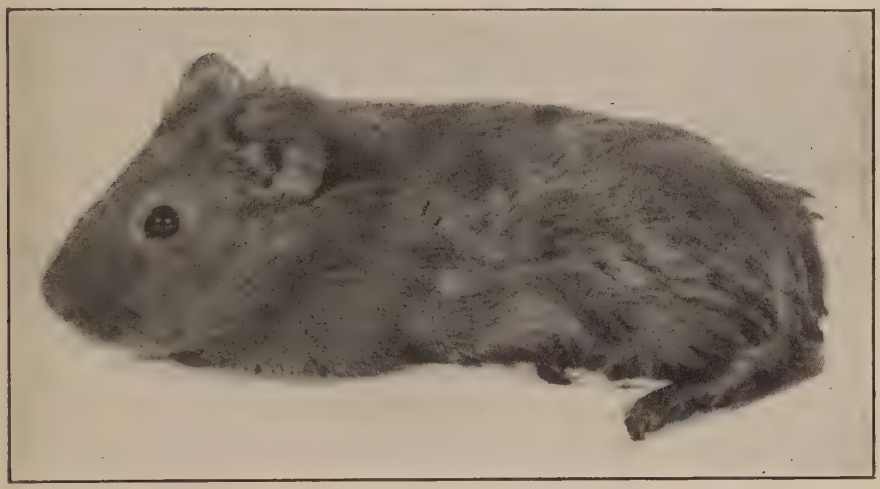


FIG. 245.—GUINEA PIG WITH SCURVY. SHOWING "SCURVY POSITION." (Hawk, Smith and Bergeim: Unpublished data.)

from which to draw his antiscorbutic vitamin. Particularly is this true of *pasteurized* milk. In the process of pasteurization the temperature to which the milk is heated destroys part of the quota of this vitamin. Fresh, raw milk has a higher content of vitamin C than milk

FIG. 246.—GUINEA PIG WITH SCURVY. SHOWING "FACE ACHE POSITION." (Special Report British Medical Research Committee, No. 38, 1919.)

which has been pasteurized.[1] This does not mean that we should prefer raw, whole milk to the pasteurized product. The proper dietetic procedure is to use pasteurized milk because of its inherently high nutritive properties and the absence of harmful bacteria and to draw

[1] Dutcher, *et al.: Jour. Biol. Chem.*, 45, 119, 1920.
Hart, Steenbock and Ellis: *ibid.*, 42, 383, 1920.

our antiscorbutic protection from other sources, *e.g.* orange juice, tomatoes, etc.

Whole milk powder, pasteurized *in the absence of air*, after condensation, has been shown to possess the same antiscorbutic properties as the raw milk from which the powder was made.[1]

Nature and Properties of Vitamin C.—Vitamin C is the most unstable of the vitamins, being readily destroyed by high temperatures and oxidation. In the absence of oxygen the vitamin is not as readily destroyed. Kohman, Eddy and Carlsson[2] have shown this point very nicely in the case of apples. In the older methods of canning as well as in the baking of apples and the preparation of applesauce the vitamin C is practically all destroyed. When apples are canned after covering them long enough with salt solution to enable the respiratory process *to use up all oxygen in them*, they may be canned without apparent loss of vitamin C. When such canned apples were placed in cold storage they showed no loss of vitamin C potency after a period of 8 months, whereas *fresh* apples gave evidence of a marked deterioration in vitamin C content after a cold storage period of about four months.

It has been shown that modern canning procedures are far less destructive of vitamin C than are the ordinary kettle cooking procedures as practiced in the average household.[3] In the case of spinach, for example, the canned vegetables may be heated to 115°C. for 1 to 2 hours and still have a product several times richer in vitamin C than the kettle-cooked article. After 9 months canned spinach was still a remarkably potent source of vitamin C. Canned cabbage has been autoclaved for 30 minutes at 100°C. and canned peas for 50 minutes at 120°C. without materially lowering their vitamin C value.[4] Bezssonov[5] has prepared a vitamin C *concentrate*, using cabbage juice as the source of the vitamin. He obtained it in the form of a slightly yellowish hygroscopic powder which protected guinea pigs in daily doses of 0.1 gram.

Detection and Estimation of Vitamin C.—The only procedure which will yield reliable data as to the antiscorbutic value of any food product is the biological assay using the guinea pig as the experimental animal (see p. 833). Bezssonov[6] has suggested a color reaction for the detection of C. It is not believed to be specific for this vitamin. Koopman[7] has attempted to correlate the results from the biological assay and the

[1] Cavanaugh, Dutcher and Hall: *Jour. Ind. Eng. Chem.*, 16, 1070, 1924.
[2] Kohman, Eddy and Carlsson: *Jour. Ind. Eng. Chem.*, 16, 1261, 1924.
[3] Eddy, Kohman and Carlsson: *Jour. Ind. Eng. Chem.*, 17, 69, 1925.
[4] Kohman and Eddy: Proc. Am. Chem Soc., *Science*, 62, 140, 1925.
[5] Bezssonov: *Bull. soc. chem. biol.*, 4, 83, 1922.
[6] Bezssonov: *Compt. rend.*, 173, 466, 1921; *Bioch. Jour.*, 18, 384, 1924
[7] Koopman: *Ned. Tijd. Gen.*, 67, 2595, 1924.

color test on the same products and claims to have secured agreement in every case except with milk. With milk he secured a negative Bezssonov reaction but the material nevertheless produced scurvy in guinea pigs.

f. Vitamin E.[1]—This vitamin was originally called vitamin X and is a *reproductive vitamin* or *fertility factor*. Evans and Bishop[2] first called attention to the fact that rats reared on certain synthetic food mixtures made up of protein, fat and carbohydrate in relatively pure form, together with proper mineral salts and adequate quantities of the vitamins A and B, sooner or later became sterile, in spite of the fact that they grew well and were apparently healthy. It has become apparent that this sterility is a dietary deficiency disease "*for it can be cured or prevented by a change in dietary regimen, a change involving the addition of certain single natural foods high in a new food factor, vitamin E*[1] *or the addition of very much smaller amounts of extracts of these foods.*"[3] Males and females are affected differently by this sterility disease. In the males the disease causes destruction of the germ cell whereas in the female the ovary and ovulation are normal but the developing young die and are resorbed.

Sure[4] has described a form of sterility which he terms *mineral sterility*. He claims that this type of sterility may be encountered when rats are fed a diet containing skimmed milk powder (prepared from winter milk) and 3 per cent of wheat oil, and may be prevented by the addition of certain mineral salts to the diet. This same investigator has also offered evidence which he interprets as indicating that wheat oil contains *two* fat-soluble dietary factors, one possessing anti-sterility properties and the other possessing lactation-promoting properties.[5]

Sources of Vitamin E.[6]—The richest source of this vitamin is the *oil of the wheat germ*. As small an amount as 0.250 gram of the dry germ given daily to an albino rat cures the sterility disease. If this desiccated germ be extracted with ether the vitamin E is removed and one drop of the resulting oil (0.025 gram) has the same curative effect as ten times that quantity (0.250 gram) of the original wheat germ.

[1] The latest of the important papers on this vitamin was by Mattill and Clayton (*Jour. Biol. Chem.*, 68, 665, 1926). An excellent bibliography is included. Mattill and associates have done much to advance our knowledge of this important vitamin.

This name was given to the vitamin by Sure (*Jour. Biol. Chem.*, 58, 693, 1924; also 62, 371, 1924), who has been active in the investigation of the relationship of this vitamin to reproduction.

[2] Evans and Bishop: *Science*, 56, 650, 1922; *Jour Am. Med. Assn.*, 81, 889, 1923; *Anat. Rec.*, 27, 203, 1924.

[3] Evans: Lectures on Nutrition (Mayo Foundation Lectures), Saunders. Philadelphia 1924–25, page 209.

[4] Sure: *Jour. Biol. Chem.*, 69, 41, 1926.

[5] Sure: *Jour. Biol. Chem.*, 69, 53, 1926.

[6] Evans and Burr: *Anat. Rec.*, 29, 356, 1925.

The vitamin is present in seeds and green leaves, the leaves of *lettuce* being particularly potent. Other sources are *alfalfa, pea leaves, oats, corn, cocoanut oil, olive oil, cottonseed oil, corn oil, peanut oil. Hemp seed* is also a good source of E. Animal tissues are notably lacking in this vitamin. Butter, cream and whole milk contain E in minimal amount.

Nature and Properties of Vitamin E.—In common with A and D, vitamin E is a *fat soluble* vitamin. It is stable to heat, light, air and many of the ordinary chemical reactions. The charring of wheat germ by heating to 170°C. does not destroy the E. The vitamin is also unimpaired after aeration of wheat germ oil for 12 hours at 97°C. It is also very stable to acid and alkali. Hydrogenation at 75°C. in the presence of palladium does not decrease its activity. It is destroyed by ultra violet irradiation for one hour.

Evans and Burr[1] have prepared a vitamin E "concentrate" of high potency from the oil of wheat germ. In common with vitamins A and D from cod liver oil the vitamin E resists saponification and is present in the non-saponifiable portion of the germ oil. The purified material is free from ash, nitrogen, sulphur, phosphorus or halogens. Its high potency is shown by the fact that it requires only 5 mg. per day to cure sterility in the female. The material may be fed to the sterile animal or introduced subcutaneously. It has also been shown that 0.3 mg. per day will cause an albino rat (male) to remain fertile throughout life when fed upon a purified synthetic diet with this small amount of material as the sole source of vitamin E.

g. Vitamin D (Antirachitic Factor).—This vitamin or *antirachitic factor* is present in highest concentration in cod liver oil. The standard foods of man are notably deficient in the content of vitamin D (see table p. 804). Foods which contain it include *milk, butter, eggs, egg yolk, lettuce, dandelion greens*, and *bread made with milk*. Because of the relatively low antirachitic potency of these foods, the only way to cure rickets or to prevent its onset is by the administration of cod liver oil or through the production of the antirachitic factor by ultraviolet irradiation (see p. 824).

Rickets.—The condition known as rickets[2] has been recognized for a long time by the obvious deformities it produces, but its accurate diagnosis was difficult and confusing until the advent of the X-ray. A characteristic of rickets is its seasonal incidence. Ordinarily, in this country rickets is rare during the summer months. It makes its appearance in the fall and has its peak in March. Then as the summer

[1] Evans and Burr: *Anat. Rec.*, 27, 203, 1924.

[2] For an extensive discussion of rickets see Park, *Physiol. Reviews*, 3, 106, 1923, and Hess, *Jour. Amer. Med. Assn.*, 78, 1177, 1922.

approaches spontaneous healing may set in.[1] A great many cases of mild rickets heal spontaneously and show little or no deformities. Deformities which persist are most commonly enlarged epiphyses of wrists and ankles. The chest and spine are affected in the more severe cases.

In most countries active rickets is restricted to the first two years of life. The deformities found later are a consequence of the rachitic process in infancy. In some localities, notably Russia, late rickets up to the age of 12 years has been described.

Mellanby[2] observed the occurrence of rickets in dogs on deficient diets. He attributed it to lack of vitamin A. The work on dogs, however, has never led to the clear cut results that have been obtained with rats. A little later, in 1922, rickets was produced in rats independently by two groups of workers, in the Medical Schools of Johns Hopkins and Columbia Universities. The former group first held that the vitamin A played a role in its production. They stated that rickets is produced by lack of vitamin A and a mineral imbalance. The Columbia group from the beginning centered its attention on the mineral components of the diet. This viewpoint was taken because it was apparent that the rachitic diet, high in Ca and low in P, could always be made non-rachitic by the addition of phosphate. Hess had already shown that vitamin A in the form of spinach had no effect on the rickets of children and Hess, Pappenheimer and McCann had produced evidence that in experimental rickets the vitamin A played no role. It was then also shown by both groups that a second type of rickets, histologically different from the usual type (such as occurs in children and in rats on a low P diet), can be produced by a diet low in Ca[3] and high in P. This type cannot be demonstrated by X-ray since there is no marked widening of the zone of uncalcified cartilage. The subject of the experimental production of rickets can be summarized by saying that rickets is produced in rats by diets low in P and high in Ca, or low in Ca and high in P, and that it can be prevented either by restoring the deficient mineral element to normal, or by giving cod liver oil. Moreover, it has been demonstrated that the action of certain wave lengths of ultraviolet light also prevents rickets (see p. 828).

The yolk of eggs contains a significant amount of antirachitic substance in the nonsaponifiable fraction of the yolk oil (Hess). McCollum produced some healing with butter fat, but the effect is very slight and has not been obtained by others.

[1] In London active rickets during the summer months is not rare and there is often no spontaneous healing in summer.

[2] Mellanby: Lancet, I, 407, 1919.

[3] Diet 85C of Pappenheimer, McCann and Zucker.

In infantile rickets and the low P type of experimental rickets, the inorganic P of the blood is lower than normal for the young animal or infant. This was first pointed out by Iverson, and in this country by Kramer. It has since been used a great deal as a means of diagnosis. A seasonal variation of the blood phosphate in infants has also been shown by Hess and Lundagen. This may be due to the seasonal incidence of rickets, but it has also been claimed that independently of rickets (in adults) there is a seasonal variation.

The normal inorganic P of the blood of infants is 4.0 to 5.0 mg., per 100 c.c. Moderate rickets show 3.0 to 3.75 mg., and in marked rickets it may fall as low as 2.0 mg. In human adults the inorganic P is 3.0 to 4.0 mg. per 100 c.c.[1]

In normal rats about 8 weeks old the inorganic P is usually 5 to 8 mg. per 100 c.c., whereas in rachitic rats it runs from 2 to 5 mg. In rats the inorganic P of the blood is influenced markedly by diet. In both infants and rats there is a marked parallelism between the degree of rickets and the inorganic P in the blood. Exceptions have, however, been noted. Healing is accompanied by, but usually precedes, a rise in P. Apparently, as long as there is still a rachitic condition, the P is low.

That the process of calcification is dependent on the inorganic composition of the bathing fluid (plasma) has been demonstrated by the work of Shipley and Howland.[2] Calcium deposition, identical histologically with that which occurs during the healing of rickets, was produced *in vitro* by immersing the bones in normal serum or in solutions simulating serum in their calcium and phosphorus contents. Best results were obtained when normal conditions of temperature and hydrogen ion concentration were maintained.

Various views have been held with regard to the etiology of rickets. It has been attributed to overfeeding in general and to a preponderance of carbohydrate. Its connection with general undernourishment has not had very many advocates, since many markedly rachitic children are well nourished and plump. Infection has been held responsible for its incidence and in France, for a long time, it was looked upon as a syphilitic lesion. Confinement and generally poor hygienic conditions as causes of rickets have held the attention of some British investigators who have also claimed curative effects from exercise and massage.

The usual view now held with regard to the etiology of rickets is that a vitamin deficiency plays a role. McCollum called this factor a vitamin (D) since, after its vitamin A had been destroyed by oxidation, cod liver oil retained its antirachitic potency.

[1] Howland and Kramer: *Am. Jour. Dis. Child.*, 22, 105, 1921.
Hess and Gutman: *Jour. Amer. Med. Assn.*, 78, 29, 1922.

[2] Shipley and Howland: Proc. Soc. Hyg., *Am. Jour. Hyg.*, 6, 314, 1926.

It does not matter whether the antirachitic substance in cod liver oil is called a vitamin or not, but the question of interest is the etiology of rickets. If we assume an antirachitic vitamin, then by definition rickets is a vitamin deficiency disease. This point of view is not universally accepted.[1]

It has been assumed that the distribution of the antirachitic factor is about the same as that of vitamin A. However, this is not the case since experimental data show that green leaves, such as spinach and lettuce, although possessing a high vitamin A potency, do not prevent or cure rickets in rats when given *ad lib.* with a rickets-producing diet. Milk and milk products (*e.g.*, cream and butter), although occupying a prominent place as foods for the growing organism, nevertheless, possess no marked antirachitic properties. Although cod liver oil had long been known as a "tonic," "flesh-builder," etc., and as a preventative or cure for rickets, little was known regarding its active components or its mode of action until recently. A very significant early investigation was that of Shabad, who showed that in rickets the phosphorus and calcium are increased in the feces and decreased in the urine, and that the administration of cod liver oil causes these elements to increase in the urine and decrease in the stools. This work shows conclusively the relation between cod liver oil and mineral metabolism. According to Zucker, Johnson and Barnett, the essential thing in the low P rickets diet is that besides low P, the Ca should be high and the reaction potentially alkaline. A more acid reaction will make such a diet non-rachitic and certain non-rachitic diets can be made rachitic by making them alkaline. Furthermore, it has been shown by Zucker and Metzner, and verified by Abrahams and Miller, that the intestinal reaction is turned in an acid direction by cod liver oil or light. Taking all these things into consideration, together with Shabad's work, it seems possible that the occurrence of rickets may be a matter of solubility of Ca and P in the intestine, the H ion concentration being largely the controlling factor. This hypothesis refers to the diets low in P, where P is the limiting factor in the rachitic process. How a similar course of reasoning might apply to calcium where it is the limiting factor is indicated by the work of Irving[2] which relates Ca absorption directly to solubility of the Ca salts in the intestine.

Funk attempted to isolate the active principle of cod liver oil in 1915. He assumed that it was a nitrogenous base (vit-amine) and followed in general a procedure applied earlier by Gautier and Mourgues[3]

[1] See Berg, "Vitamins—A Critical Survey of the Theory of Accessory Food Factors," Knopf, New York, 1923.

[2] Irving: *Jour. Biol. Chem.*, 68, 513, 1926.

[3] Gautier and Mourgues: *Compt. rend. Acad. Sci.*, 107, 110, 254, 626 and 740, 1888.

and by Hawk[1] for isolating bases from oils. These products were never shown to be antirachitic.

Zucker approached the problem in another way. The oil was divided into fatty acids and nonsaponifiable fraction. The fatty acids were inactive; the nonsaponifiable fraction was active. In this fraction it was not organic bases or cholesterol that was active, but a cholesterol-like residue which could be concentrated by removing more and more cholesterol and other impurities. Another method of obtaining the active substance in quantity is, first, to extract the oil with an immiscible solvent in which the active substance is soluble, such as alcohol, then to discard the extracted oil and saponify the alcoholic extract. To avoid extraction of a sodium soap solution the latter is precipitated as calcium soap which carries down the antirachitic substance. From the calcium soap it is extracted with acetone and this extract further purified. Further work along this line has been done by Drummond, Takahashi and Dubin.

For a discussion of the relationship of ultraviolet light to the cure and prevention of rickets, see page 828.

Shear Color Reaction for Vitamin D.[2]—Add 1 part of concentrated HCl to approximately 15 part of aniline. Intoduce 3 c.c. of cod liver oil or the solution under investigation, into a wide test tube and add an equal volume of the aniline reagent. Mix the contents of the tube thoroughly, heat to boiling with constant shaking and boil for about half a minute. The yellow emulsion should turn green and in a few seconds change to red. Within a minute or two the emulsion should separate into two layers, the lower one being colored an intense red. On standing the red color deepens and persists.

Certain oils which give no red color with this test have been shown to give a positive reaction after irradiation with ultraviolet light.

Shear believes that this test is given by vitamin D and that there may be a close connection, if not an identity, between the antirachitic factor and the chromogenic substance. Another color reaction for vitamin D using phosphomolybdotungstic acid has been described by Bezssonov.[3] Rosenheim and Webster report that these color tests are not specific for vitamin.[4]

ULTRAVIOLET RADIATION

Nature of Radiation.—The visible spectrum (see Frontispiece) is only a small part of the entire color spectrum. Radiant energy of the same physical characteristics and differing only in wave length and in frequency of vibration, includes not only visible light, but ultraviolet

[1] Hawk: *Am. Jour. Physiol.*, 21, xxii, 1908.
[2] Shear: *Proc. Soc. Expt. Biol. Med.*, 23, 546, 1926.
[3] Bezssonov: *Comptes Rendus*, 179, 572, 1924.
[4] Rosenheim and Webster: *Bioch. Jour.*, 20, 537, 1926; 20, 544, 1926.

and infrared radiation, electric waves of a great range of wave lengths, X-rays and the gamma rays of radiant matter.

In 1868, Maxwell announced the hypothesis that light is an electromagnetic phenomenon. In place of ether vibrations emanating in concentric spherical waves in all directions, the electromagnetic theory predicates the existence of electrical waves, which may be longer than those in the visible and infrared regions.[1] Hertz, in 1888, verified this prediction, and in 1895 Röntgen discovered the X-rays which were later classified as radiations of short wave lengths. The subsequent research of other investigators, has resulted in bridging the gap between the ultraviolet and the X-ray spectra, by overlapping spans projected from either side.

All radiations have a common velocity of propagation about 300,000 kilometers per second (186,000 miles), but each is defined by wave length or frequency of pulsation. Since the velocity of transmission is the same for all waves and a wave length is traveled in 1 period, wave length is proportional to the length of period and inversely proportional to the frequency. The frequency is obtained by dividing the velocity by the wave length. Hence the smaller the wave, the more waves in a second and to a centimeter in the line of travel.

Three terms and symbols are in common use for designating wave lengths. Their relations and magnitudes are as follows:

Unit	Symbol	Millimeters	Relative length
Angström	Å	one ten millionth	1
Millimicron	mμ or μμ	one millionth	10
Micron	μ	one thousandth	10,000

For example, the approximate boundary between the visible and ultraviole tregions of the spectrum is expressed in the three units as follows: 4000Å; 400 mμ; 0.4μ. This corresponds to the frequency of

[1] While the electromagnetic theory still remains, the undulatory or wave theory has been seriously challenged by Planck's theory of energy quanta in radiation and the relativity theory of Einstein. It is now believed that radiation is that form of pulsating energy emitted whenever an atom disintegrates; or, in other words, it is the result of internal cataclysm of the atom. The electrons of the atom revolving around the "nucleus" much as the planets revolve about the sun, are in some way, not clearly defined, caused to drop from one orbital path into another closer to the nucleus. Whenever this happens, energy is emitted. The excess of energy which is hereby lost to the atom is shot out into space in perfectly definite quantities—"light quanta." If a great deal of energy is emitted, it has a rapid vibration frequency and is of the nature of ultraviolet or X-rays, if the amount is smaller, infrared or Hertzian rays are the result. The quantum theory is the basis of Einstein's theory of the transformation of radiant to chemical energy. Thus we are returning to the old Newtonian emission theory, but with the vast difference that electromagnetic energy, instead of matter, is emitted from the source in all directions.

73×10^{13} per second. The accompanying diagram shows roughly the approximate wave length limits in $\mu\mu$, of the various spectral regions of radiant energy.

Infrared red (invisible)	Visible	Ultraviolet (invisible)
Field of Hertzian waves	red green blue indigo violet	X-ray radium
12 Km + 1000 900	800 700 600 500	400 300 200 100 0

Sources of Ultraviolet.—Ultraviolet radiation can be obtained from many sources to meet varying conditions. The ideal source for scientific investigation is one emitting a continuous spectrum of high and uniform intensity, but this does not exist for any low range in the ultraviolet region. Discharge tubes, sparks, arcs, incandescent solids, and combinations of these can all be used, but the quartz mercury vapor burner and the carbon arcs lend themselves most readily to, and have been most extensively used in, biological work.

The burner of the quartz mercury vapor lamp consists of a fused quartz chamber with the arc discharge taking place either between two electrodes of liquid mercury, or a mercury cathode and a tungsten anode. The spectrum is discontinuous, with a diffuse continuous spectrum superimposed upon the lines.

Sources differ not only in the distribution, but also in the intensity of the wave lengths of the radiant energy they emit. The ultraviolet component of a mercury vapor lamp decreases in intensity as the lamp is burned. With increase in energy input the percentage of infrared rises and there is considerable infrared present as a result of heating of the electrodes and the supports of the lamp. Thus the ultraviolet component is only about 20 per cent of the total radiation of all wave lengths emanating from the whole lamp.

The sources most commonly used in this country are the Hanovia (see Fig. 247) and Copper-Hewitt quartz mercury arcs. They can be obtained either in the air cooled or the water cooled models, the former being preferable for experimental work because of its simplicity in operation.

The sun is very rich in visible and infrared wave lengths, but has relatively little ultraviolet. On a clear day at sea level the distribution is approximately 1–2 per cent of ultraviolet, 40–50 per cent of visible, and 50–60 per cent of infrared rays.

The ultraviolet from the sun and a new 110 volt quartz mercury vapor lamp at 30 cm. (1 ft.) are roughly the same in intensity, but it

must be borne in mind that the spectral quality (energy distribution) is entirely different—the quartz mercury vapor lamp being relatively stronger in radiation of wave lengths which are lacking in the solar spectrum.

There is a marked physiological difference between the effect of light from 400 to 300 mμ (near ultraviolet) and the light from 300 to 200 mμ (middle ultraviolet). "Light of greater wave length than 300 mμ being our normal environment, it is obvious that any organism

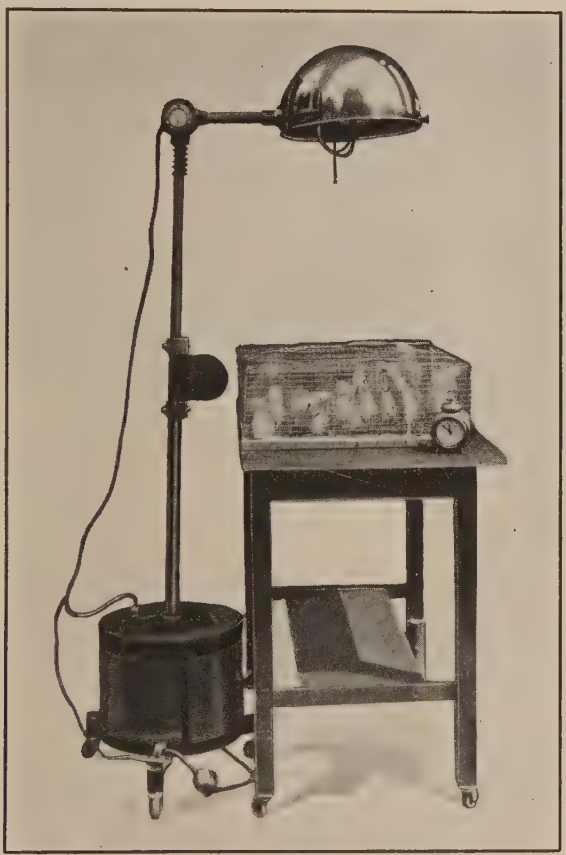


FIG. 247.—IRRADIATION OF ALBINO RATS (HANOVIA QUARTZ MERCURY VAPOR LAMP).

ordinarily exposed to this light and easily injured by it, would long since have perished. Light less than 300 mμ is unnatural and produces in all living cells strong and often harmful reactions."

It is thus seen that different sources vary markedly in their respective components of ultraviolet, visible, and heat rays, and that it is essential in the proper use of radiation from the sun or artificial sources to measure accurately the energy content in order to determine the

dosage. Many of the conflicting results obtained in the past are probably due to the neglect of this factor.

The methods for measuring the intensity of radiation may be divided into two classes: (1) those designed for rapid and easy use, which make use of biological or photochemical reactions to determine the entire ultraviolet component as such, and (2), the more accurate photometric and radiometric methods. A description of these methods is outside the scope of this book. Suffice it to say, however, that while the lithopone method of Clark,[1] and the acetone oxidation method of Webster, Hill and Eidenow,[2] are sufficiently accurate for clinical use they simply serve to gauge the gross intensity of total ultraviolet present and do not indicate the percentage distribution or the relative intensity of the wave lengths in that region. Experimental laboratory research demands much more accurate methods, such as those mentioned in (2) above.[3]

A useful method of standardizing ultraviolet lamps for clinical use has been described by Pohle.[4]

Physiological Relationships of Ultraviolet Light.—A most important phase of the study of rickets is its relation to certain wave lengths of light. Huldschinsky[5] first demonstrated the remarkable effect of light on rickets. Certain wave lengths (about 300$\mu\mu$) will affect the mineral metabolism of infants so as to prevent or cure rickets. Hess, Unger and Pappenheimer[6] then showed that this holds also for experimental rickets in animals. Much work is being done on the influence of ultraviolet light,[7] particularly its application to the treatment of infantile rickets. In the clinical treatment of rickets, however, ultraviolet light has not yet achieved the success which follows the administration of cod liver oil. An interesting development is the activation of certain sterols (cholesterol, phytosterol, etc.) or sterol-containing foodstuffs by these same wave lengths. For example eggs, milk, spinach, lettuce etc., have been shown to develop pronounced antirachitic properties upon irradiation. When such irradiated products are fed with rachitic diets they prevent rickets just as if the animal itself were irradiated. Excessive irradiation of foods will destroy vitamin A.[8] Maslow, Shuling and Kramer[9] have shown that irradiated orange juice has important antirachitic properties, preliminary healing of rickets being demonstrated in five days and *almost complete cure* in fifteen days.

[1] Clark: *Am. Jour. Physiol.*, 69, 200, 1924.

[2] Webster, Hill and Eidenow: *Lancet*, I, 745, 1924.

[3] The thermopile-galvanometer method is often used for accurate experimental work. It possesses the advantage over the others in that the thermopile is non-selective, is relatively easily controlled and is in general, more reliable. The absorbed energy acting on a thermo-electric couple causes a flow of current which deflects the needle of a sensitive galvanometer in circuit with it. *The value of a unit* galvanometric deflection is then determined in terms of a radiation standard. Since the energy value of the latter is known, the value of the radiation intensity of any unknown source is measured by the galvanometric deflection which it produces.

[4] Pohle: *Am. J. Roentgenology and Rad. Ther.*, 15, 193, 1926.

[5] Huldschinsky: *Deut. Med. Woch.*, 45, 712, 1919; *Zts. f. orthopaed. Chir.*, 39, 426, 1020.

[6] Hess, Unger and Pappenheimer: *Jour. Exp. Med.*, 36, 427, 1922.

[7] See Park: *Physiol. Reviews*, 3, 106, 1923 for bibliography.

[8] Titus *et al*: *Ind. Eng. Chem.*, 18, 843, 1926.

[9] Maslow, Shuling and Kramer: *Bull. Johns Hopkins Hosp.*, 39, 56, 1926.

The irradiation of cows, chickens etc., see Fig. 248, has also met with a measure of success. It is believed the ultraviolet light exerts its curative action in rickets through the activation of the cholesterol of the skin, thus producing from it the antirachitic factor. The effect of ultraviolet light on growth is a subject which is being actively studied. Certain investigators, (Laurens and Sooy, Hume and Steenbock and associates) believe that the ultraviolet rays are necessary for growth. Other workers (Goldblatt and Soames, Sheets and Funk) report that ultraviolet irradiation does not stimulate growth.

Studies from the Laboratory of Physiology of Yale University (Mayerson, Gunther and Laurens)[1] have shown that dogs kept in the dark for long periods

FIG. 248.—PROGRESSIVE WEAKENING OF LEGS OF A CHICK DEPRIVED OF RADIANT ENERGY, AND ON A DIET LOW IN PHOSPHORUS AND ANTIRACHITIC FACTOR. CONTROL, UPPER LEFT, HAD SAME DIET BUT OCCASIONAL SUNSHINE. (Price: *Ind. Eng. Chem.*, 18, 679, 1926.) IRRADIATION OF HENS HAS ALSO BEEN EMPLOYED TO INCREASE THE ANTIRACHITIC FACTOR IN THE EGG YOLK.

acquire a lowered resistance whereas irradiation of normal animals causes a distinct variation in their metabolic response. It seems to the authors on the basis of a rather limited experience with ultraviolet irradiation, that the influence on growth is quite dependent upon the dosage. The fact that different investigators report varying findings may possibly be due to the fact that irradiation procedures are not standardized. Observers in different laboratories use different procedures (time, voltage, distance, etc.) and must inevitably arrive at different conclusions. We are inclined to favor the view that the proper dosage of ultraviolet promotes growth (see page 810). It is possible, as claimed by Eckstein, that overirradiation may stunt the growth.

EXPERIMENTS ON ULTRAVIOLET IRRADIATION

1. Influence of Ultraviolet Light in Rickets.—Take two white rats from one to two months old (of same litter) and weighing 30–60 grams each. Introduce them into individual cages (see p. 793) and feed them the Steenbock rachitic diet No. 2965 (see p. 835). Keep one rat in the dark or in a poorly lighted room.

[1] Mayerson, Gunther and Laurens: *Am. Jour. Physiol.*, 75, 399 and 421, 1926.

Keep the other in direct sunlight (not behind glass) for 30 minutes or more each day. A three minute exposure to the rays of an ultraviolet (quartz mercury vapor) lamp (see p. 827) at a distance of 3 feet may be substituted. At the end of one month examine both animals radiographically. The animal kept in the dark will have contracted rickets (see Fig. 256, p. 837) whereas the animal exposed to the light will remain normal (Fig. 257, p. 837). Reverse the experimental conditions for the two animals and examine radiographically at the end of another month. By this time the rachitic animal will probably have been cured, whereas the normal animal will have contracted rickets. The animals may now be autopsied, and the X-ray findings checked by the "line test" (see p. 838).

2. Antirachitic Activation of Foodstuffs by Ultraviolet Light.[1]—Take two white rats from one to two months old (of the same litter) and weighing 30–60 grams each. Introduce them into individual cages (see p. 793) and feed one of them the Steenbock rachitic diet #2965 (see p. 835) and the other this same diet which has been irradiated.[2]

At the end of one month, examine both animals radiographically (knee-joint) and compare the findings with Figs. 256 and 257, page 837. The animal receiving the non-irradiated diet will have contracted rickets and the radiograph will show a band of uncalcified cartilage near the ends of the tibia and femur (Fig. 256). The bones of the animal receiving the irradiated diet will remain normal (Fig. 257) due to the production of the antirachitic factor by the ultraviolet light.

In order to make a more complete experiment, the diets may be reversed, and after a proper interval (one month or longer) the rachitic animal may be shown to be cured, whereas the normal animal will have become rachitic. If so desired the animals may be autopsied, the tibias removed and the "line test" (see p. 838) performed.

EXPERIMENTS ON DIETARY DEFICIENCIES AND VITAMINS

Approximate weight equilibrium is a standard condition for the majority of adult men and women. The child, on the contrary, must show a continuous gain in body weight in order to be adjudged normal. All other things being equal, the child who receives an adequate diet will show normal growth gains, whereas the child who receives a deficient diet will fail to grow or will grow at an abnormally slow rate. Likewise with the adult, the proper nutritive substances

[1] Steenbock, at the University of Wisconsin, and Hess, at the College of Physicians and Surgeons, New York, independently demonstrated the antirachitic activation of foodstuffs by ultraviolet radiation. Steenbock's paper was received by the Journal of Biological Chemistry in April, 1924 (Steenbock and Black, *Jour. Biol. Chem.*, 61, 405, 1924) and published in the September issue, an earlier announcement appearing in *Science*, September 5, 1924. Hess made a preliminary report in June, 1924 (*Proc. Am. Pediat. Soc.*, June 7, 1924, abstracted in *Am. J. Dis. Child.*, 28, 256, 1924) and his experiments were received for publication by the Journal of Biological Chemistry in September and appeared in the December issue (Hess and Weinstock, *Jour. Biol. Chem.*, 62, 301, 1924). Thus Steenbock may be credited with the first published statement and Hess with the first public announcement of their findings. However, since these investigations were conducted independently credit should perhaps be equally divided.

[2] A Cooper-Hewitt or Hanovia quartz mercury vapor lamp run at 120 volts may be used. In irradiating the ration it should be exposed at a distance of two feet for 10 minutes in 50 gram portions, spread out in a *thin layer* in a 10 inch porcelain dish. Irradiate a fresh supply every day, feed immediately after irradiation and discard all unconsumed residues from the previous feeding. (Steenbock and Black: *Jour. Biol. Chem.*, 61, 405, 1924.)

must be ingested in order to afford material from which worn-out body structures may be repaired, and vitality and the proper functioning of the various organs maintained. The character and extent of the dietary deficiency will regulate the character and extent of the gains or losses in body weight and the general physical efficiency of the individual.

In order that a diet may be adequate for normal growth, and reproduction and at the same time afford adequate protection against rickets, scurvy and certain other nutritional disorders it must contain at least ten factors as follows:

1. Vitamin A (Fat-Soluble A).
2. Vitamin B (Water-Soluble B).
3. Vitamin C (Antiscorbutic Vitamin).
4. Vitamin D (Antirachitic Factor).
5. Vitamin E (Reproductive Vitamin).
6. Factor P-P (Pellagra-Preventing factor).
7. Protein, proper in kind and amount.
8. Energy furnished by fats and carbohydrates.
9. Inorganic matter, proper in kind and amount.
10. Water.

In order that the above dietary factors shall function most satisfactorily, sufficient roughage should be included to obviate constipation and insure the evacuation of normal stools.

By the use of the proper experimental animals, the influence of a diet deficient in any one of the ten essential dietary factors may be readily demonstrated. Typical dietary demonstrations of this character are outlined on the following pages.

1. Influence of Vitamin Deficiency.—For discussion of vitamin deficiency see pp. 803 to 830.

(a) Demontration on Vitamin B.—Take two white rats from one to two months old and weighing 30–60 grams each and feed them daily upon a diet adequate from every standpoint.[1] Such a diet may consist of casein (20 per cent), butter fat (15 per cent), starch (56 per cent), salt mixture (4 per cent[2]) and yeast (5 per cent). The yeast furnishes the vitamin B whereas the vitamin A is furnished by the butter fat. On the above diet the rats will show normal growth (see Fig. 249) approximately doubling their weight in two weeks.[3] The animals should be weighed about twice a week. At the end of two weeks eliminate the yeast from the diet of one of the rats leaving the diet of the other rat unchanged. The diet of one animal now lacks the water soluble vitamin and this animal will not only fail to grow but will show an actual loss of weight.

[1] Excellent *casein, salt mixture* and *yeast* preparations for work of this character may be obtained from the Harris Laboratories, Inc., Tuckahoe, New York. A very satisfactory powdered yeast preparation may also be obtained from the Northwestern Yeast Co., Chicago, Ill. A very satisfactory form of cage in use in the senior author's laboratory may be obtained from the A. B. Hendryx Co., New Haven, Conn. This cage is shown in Fig. 233, page 793.

[2] The salt mixture should have the following composition (Osborne and Mendel):

$CaCO_3$	134.8 gm.	K_2CO_3	141.3 gm.	H_2SO_4	9.2 gm.
$MgCO_3$	24.2 gm.	H_3PO_4	103.2 gm.	Citric acid + H_2O	111.1 gm.
Na_2CO_3	34.2 gm.	HCl	53.4 gm.	Fe citrate + $1\frac{1}{2}H_2O$	6.34 gm.
KI	0.020 gm.	$MnSO_4$	0.079 gm.	NaF	0.248 gm.
				$KAl(SO_4)_2$	0.0245 gm.

[3] For other normal growth curves of albino rats see "The Rat," by Henry H. Donaldson, Wistar Institute, Philadelphia, Pa.

A period of two weeks on this diet will be sufficient to demonstrate these facts. The other animal will continue to grow normally. Compare the weights of the two rats. Plot their growth curves (see Fig. 249).

The curve shown in Fig. 250, p. 833, is the growth curve of a white rat fed as just described.[1]

The rat pictures reproduced in Figs. 251 and 252 also show the influence of vitamin B in the diet. The animals each weighed about 50 grams at the

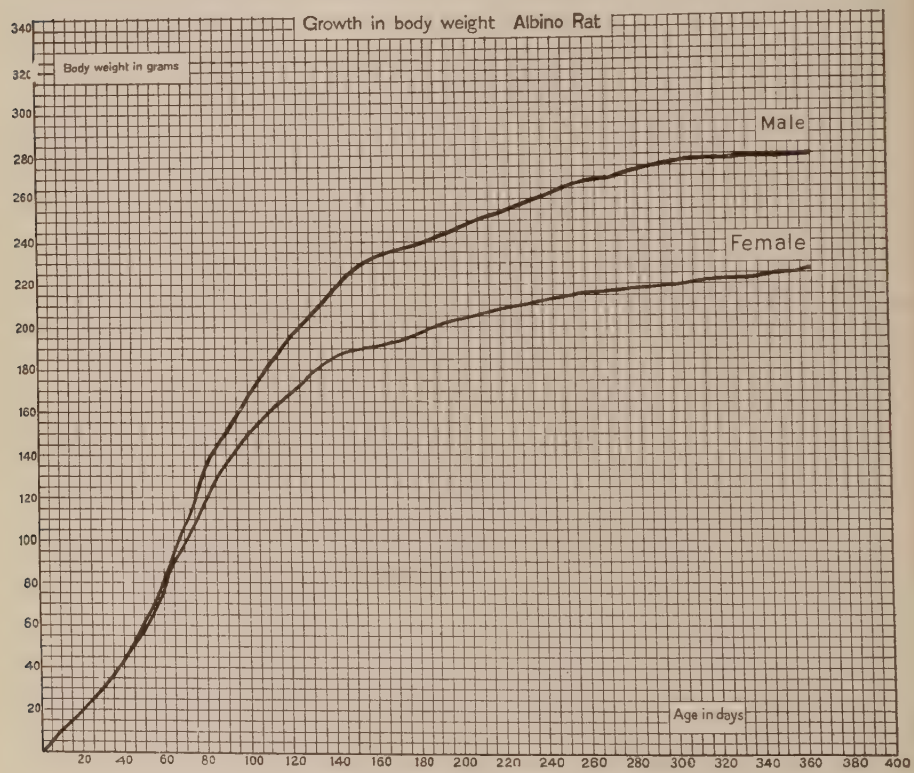


FIG. 249.—NORMAL GROWTH CURVES OF ALBINO RATS.[2]
(After Donaldson.)

beginning of the experiment. At the end of a few weeks, the rat (No. 4) receiving an inadequate supply of this vitamin showed no gain in weight, whereas the other animal (No. 14), receiving a more adequate supply of this growth-promoting substance weighed 115 grams.

An experiment similar to the above may be made by replacing the casein by "meat powder" prepared from fresh lean beef, ground and dried in a current of air at about 100°C. A typical growth curve from such an experiment[3] is shown in Fig. 253, page 835.

[1] Hawk, Fishback and Bergeim: *American Journal of Physiology*, 48, 211, 1919. See also Osborne and Mendel: *Jour. Biol. Chem.*, 31, 149, 1917; 32, 309, 1917; Funk and Macallum: *Jour. Biol. Chem.*, 23, 413, 1915; 27, 51, 1916.

[2] For a discussion of growth curves from the standpoint of the modern adequate diet see Osborne and Mendel: *Jour. Biol. Chem.* 69, 661, 1926.

[3] Osborne and Mendel: *Jour. Biol. Chem.*, 32, 309, 1917.

(b) Demonstration on Vitamin A.—In this experiment two white rats may be used as subjects. Feed them a diet similar to the one given on p. 831, and after normal growth has been demonstrated (two weeks) replace the butter in the diet of one of the rats by lard, leaving that of the other animal unchanged. The diet of one rat now contains little or no vitamin A and this rat will consequently fail to grow. The other animal will show normal growth gains. Compare the weights of the two animals. Plot their growth curves (see Fig. 249). An

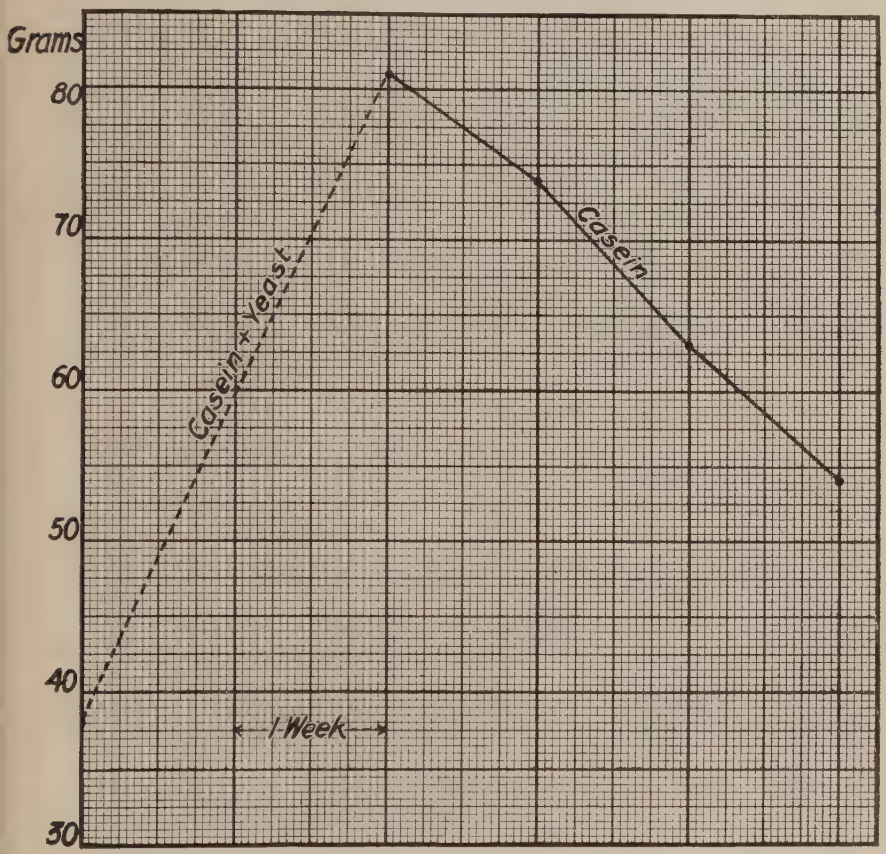


Fig. 250.—Growth Curve of Albino Rat Showing Importance of Vitamin B.

(Hawk, Fishback and Bergeim: *American Journal of Physiology*, 48, 211, 1919.)

interval of three weeks is long enough to demonstrate this point. See Figs. 254 and 255.

(c) Demonstration on Vitamin C.—Feed two guinea-pigs, weighing about 250 to 300 grams each, a diet of rolled oats, hay which has been heated at 105° C. for several hours, and water. In addition to the above give each animal 20 c.c. of pasteurized milk daily. Upon the appearance of scurvy, which should occur in from 10 days to 2 weeks, add 5 c.c. of orange juice to the daily ration of each guinea-pig, and note results. The onset of scurvy is evidenced by a variety

of symptoms chief of which are the following: The joints become tender so that the animal will wince and cry when it is examined, this tenderness being often accompanied by swelling due to edema or hemorrhage. The animal

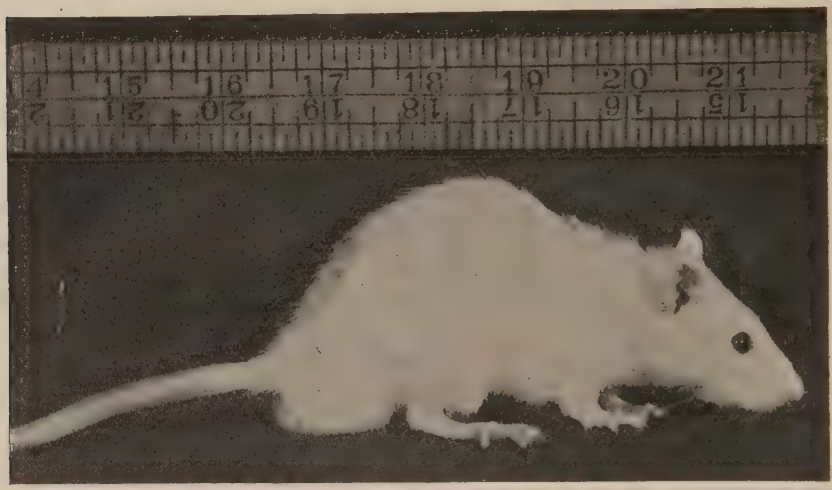


FIG. 251.—RAT (NO. 14) FED A DIET CONTAINING SUFFICIENT VITAMIN B. COMPARE FIG. 252.

(Hawk, Smith, and Bergeim: *American Journal of Physiology*, 56, 33, 1921.)

soon becomes lethargic instead of over-excitable, and generally assumes an unnatural posture such as holding up one tender hind leg, "the scurvy position," (see Fig. 245) or lying with the side of its face resting on the floor, "face ache

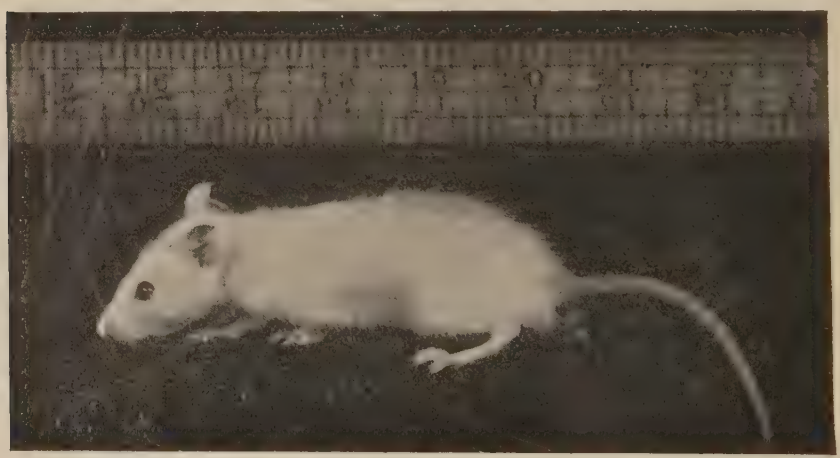


FIG. 252.—RAT (NO. 4) FED A DIET DEFICIENT IN VITAMIN B. COMPARE FIG. 251.

(Hawk, Smith, and Bergeim: *American Journal of Physiology*, 56, 33, 1921.)

position." (See Fig. 246.) A couple of days after orange juice is added to the diet, the animals should show signs of improvement. The length of time elapsing before complete recovery depends upon the individual case.[1]

[1] For further details concerning scurvy see Hess: "*Scurvy Past and Present*," Lippincott, 1920.

(d) Demonstration of the Antirachitic Factor.—Two young rats which have grown rapidly to about 55 to 60 grams (35 to 40 days old) are confined to one of the following diets for a period of 18 to 28 days (preparatory period):

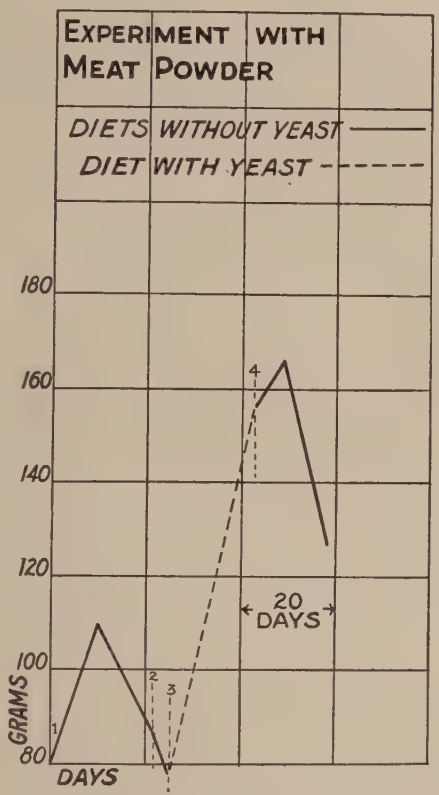


FIG. 253.—GROWTH CURVE OF ALBINO RAT SHOWING IMPORTANCE OF VITAMIN B.
(Osborne and Mendel: *Journal Biological Chemistry*, 32, 309, 1917).

RACHITIC DIETS

(Parts per hundred)

Steenbock Diet No. 2965[1]		McCollum Diet No. 3143[2]	
Yellow corn	76.0	Whole wheat kernel	33.0
Wheat gluten	20.0	Whole maize kernel	33.0
Calcium carbonate	3.0	Gelatin	15.0
Sodium chloride	1.0	Wheat gluten	15.0
		NaCl	1.0
		$CaCO_3$	3.0

[1] Steenbock and Black: *Jour. Biol. Chem.*, 64, 263, 1925.
Nelson and Steenbock: *ibid.*, 64, 299, 1925
[2] McCollum, Simmonds, Shipley, and Park: *Jour. Biol. Chem.*, 51, 41. 1922.

FIG. 254.—RAT FED A DIET DEFICIENT IN FAT-SOLUBLE A (LEFT) AND RAT FED AN ADEQUATE DIET (RIGHT).

(From McCollum and Simmonds "The Newer Knowledge of Nutrition." 3rd edition, p. 285, 1925.)

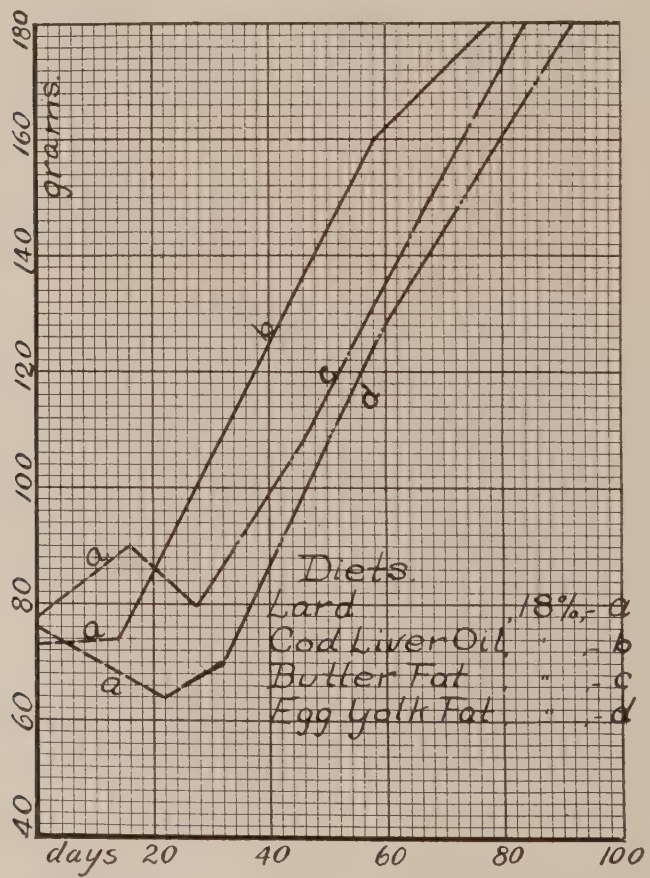


FIG. 255.—VITAMIN A AND GROWTH. THE DIET CONTAINING LARD WAS DEFICIENT IN VITAMIN A, WHILE COD LIVER OIL, BUTTER FAT, AND EGG YOLK FAT ARE RICH IN THIS SUBSTANCE.

(Osborne and Mendel: *Journal of Biological Chemistry*, 16, 434, 1913; 17, 405, 1914.)

Pappenheimer-McCann-Zucker Diet No. D[1]	
Patent Flour	80.9
Egg Albumin	10.0
Butter fat	5.0
Salt Mixture[2]	4.1

This salt mixture is based on analyses of the milk of small animals instead of cow's milk.

After 18 to 28 days on either of these diets it will be noted that the rats have some difficulty in using their hind legs. At this time radiographs may be made

FIG. 256.

FIG. 257.

FIG. 256.—ACTIVE RICKETS. Radiograph of knee joint of rat on Steenbock rachitic diet No. 2965 for 17 days. Note especially broad bands of uncalcified cartilage near ends of tibia and femur. (Unpublished data from the senior author's laboratory.)

FIG. 257.—HEALED RICKETS. Radiograph of knee joint shown in Fig. 256 after the rat had been fed a cod liver oil concentrate 14 days. (Unpublished data from the senior author's laboratory.)

of one of the hind legs of each animal (at the knee joint) and should reveal broad light bands of uncalcified cartilage at the ends of the tibias and femurs. This is the typical rachitic bone. See Fig. 256, above.

Continue the rats on the same diet except that one animal is given, mixed with its food, 3 per cent of cod liver oil. Repeat the X-ray examinations at weekly

[1] Pappenheimer, McCann, and Zucker: *Jour. Exp. Med.*, 35, 447, 1922.

[2] The salt mixture in the Pappenheimer-McCann-Zucker diet has the following composition (gm. per 100 gm. of diet):

KCl	0.85
Na_2CO_3	0.85
$MgCO_3$	0.286
Ca lactate	2.000
Ferric citrate	0.1
KI	0.0002
$MnSO_4$	0.00078
NaF	0.0024
$KAl[SO_4]_2$	0.00024
Total	4.08962

intervals. The control rat should show more pronounced rickets as time passes with a wider decalcified band as in Fig. 256. In the case of the rat given cod liver oil, as the rickets heals the band of uncalcified cartilage becomes narrower and finally disappears. At this point the bone assumes the appearance of normal bone (see Fig. 257). The experimental findings may be confirmed by performing the "line test" (see below). The rat should also show recovery from lameness.

The "Line test" for Calcium-depositing Substances.[1]—**Principle.**—A silver staining method is used to bring out the provisional zone of calcification which appears as a line of calcium salts extending transversely across the bone with the Ca-free cartilage and metaphysis on either side.

Procedure.—The tibia of the rat is dissected out and immersed in acetone for two days. The bone is then rinsed thoroughly with distilled water and split longitudinally. One half of the bone is immersed in dilute (2 per cent) silver nitrate solution and exposed to a powerful light.[2] It is then rinsed and the split surface is examined through a binocular microscope while immersed under distilled water.[3] By this treatment the calcified areas in the bone become coated with silver phosphate which the light reduces to black metallic silver. To preserve these bones, wash well with water, decolorize with a solution of sodium thiosulphate, rewash and keep in formaldehyde.

Interpretation.—The presence of a newly formed line of calcification (honey-comb structure) in the proliferative cartilage indicates healing of rickets. The line may extend completely across the bone or may be incomplete and fragmentary, according to the degree of deposition of calcium salts induced by the factor (diet, light, etc.) under investigation. See Plate VIII[4], between pages 838 and 839. Since starvation may cause a similar deposit of Ca salts in the cartilage of rachitic animals, food intake must be carefully watched.

2. Influence of Protein (Amino Acid) Deficiency.—At least four of the essential amino acids which occur in protein substances cannot be synthesized in the animal body. These are *cystine*, *lysine*, *tryptophane*, *tyrosine* and probably *histidine* and *proline* (see Chapter V). The acids mentioned must, therefore, be included in our diet if we are to be properly nourished. The importance of proper protein (amino acids) in the diet is illustrated in Fig. 258. The following experiments.

[1] McCollum, Simmonds, Shipley and Park: *Proc. Soc. Exp. Biol Med.*, 19, 123, 1921. *Jour. Biol. Chem.*, 51, 41, 1922.

[2] A 3 minute exposure to ultraviolet light at a distance of one foot from the burner, is recommended by Hess and Weinstock. Personal communication

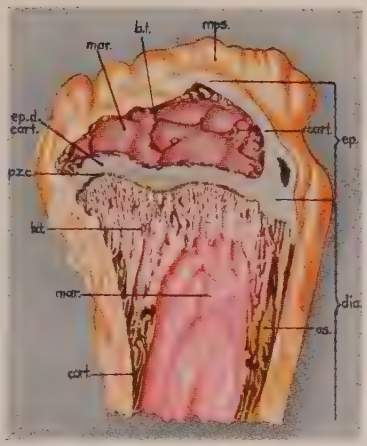
[3] Bills (Personal Communication) examines the bone directly in the silver nitrate solution, using a powerful stage illuminator which also provides actinic rays for the reduction of the silver salt.

[4] These illustrations were prepared by Mr. James F. Didusch from dissections made by Dr. Charles E. Bills and are reproduced through the courtesy of Mead Johnson and Co.

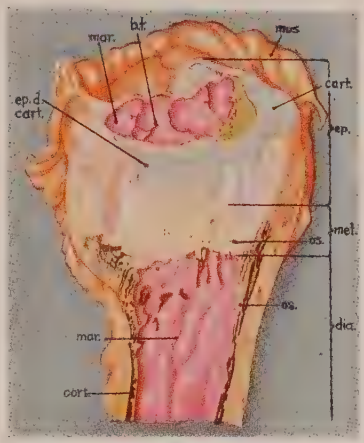
PLATE VIII

Normal Tibia, from a rat which had never had rickets.
Severe Rickets, produced by Diet 3143 (see p. 835).
+ Healing Rickets, indicating just perceptible healing.
++ Healing Rickets, indicating distinct healing.
+++ Healing Rickets, indicating advanced healing.
++++ Healing Rickets, indicating a practically complete healing, the amount of calcium being normal, and only some structural evidences of rickets remaining.

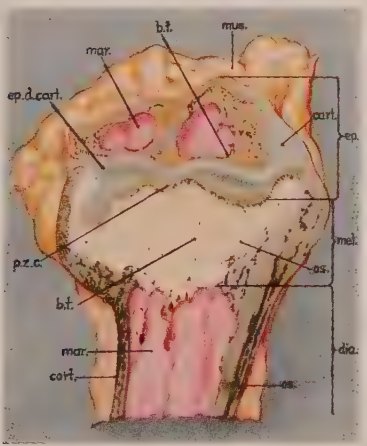
b.t. = bony trabecula
cart. = cartilage
cort. = cortex
dia. = diaphysis
ep. = epiphysis
ep.d.cart. = epiphysial disc of cartilage
mar. = marrow
met. = metaphysis
mus. = muscle, etc.
os. = osteoid
p.z.c. = provisional zone of calcification



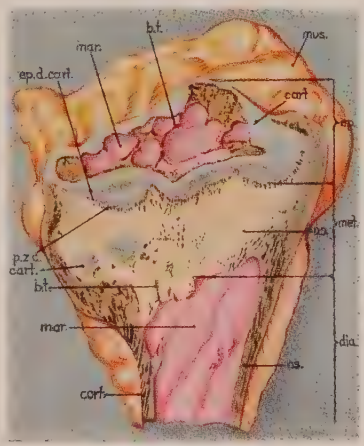
NORMAL TIBIA



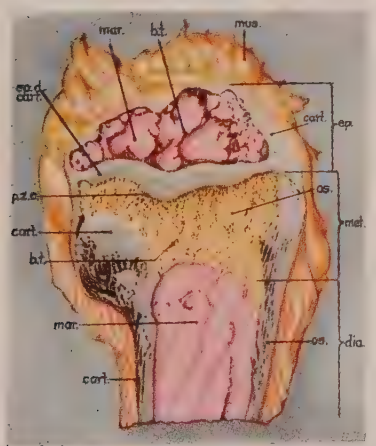
SEVERE RICKETS



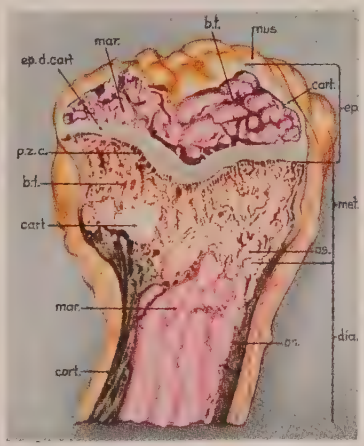
+ HEALING RICKETS



++ HEALING RICKETS



+++ HEALING RICKETS



++++ HEALING RICKETS

LINE TEST ON NORMAL AND RACHITIC TIBIAS. (*After Bills and McDonald: Jour. Biol. Chem.*, 68, 821, 1926.)

which may readily be made using white rats as subjects, will clearly demonstrate the importance of two of these amino acids, *i.e.*, cystine and lysine.

(a) Demonstration on Cystine Deficiency.—Place two young white rats (40–60 grams) in separate cages (see Fig. 233). Feed one rat Diet 1 and the other rat Diet 2 as listed in the following table:—

CYSTINE DEFICIENCY DIET

	Diet 1 Per cent	Diet 2 Per cent
Cooked Navy bean meal[1]	72.00	71.64
Cystine	0.00	0.36
Salt mixture	4.00	4.00
Butter fat	15.00	15.00
Lard	9.00	9.00

The animal receiving Diet 1 will fail to grow because of the deficiency of cystine. On the other hand, the rat receiving Diet 2 will grow normally, because cystine is present in proper amount. The rats should be weighed at short intervals. See Fig. 259.

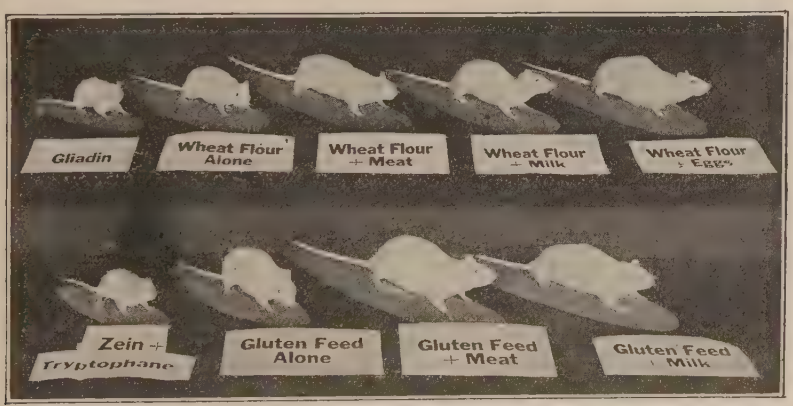


FIG. 258.—SHOWING IMPORTANCE OF ADEQUATE PROTEIN (AMINO ACIDS) IN THE DIET. (Mendel: "Nutrition—The Chemistry of Life," Yale University Press, 1923.)

(b) Biological Assay of Cystine.[2]—Principle.—Since under properly controlled experimental conditions, an increase in the growth rate can be induced by the addition of a single amino acid to the diet, this may be interpreted to indicate that there is an unused margin in the basal diet of all other essential amino acids. Different additions (below the

[1] Navy bean meal cooked 3 hours with distilled water, dried and ground (John and Finks: *Jour. Biol. Chem.*, 41, 375, 1920). Cystine may be prepared as described in Chapter V.

[2] Sherman and Woods: *Jour. Biol. Chem.*, 66, 29, 1925.

optimum) of the limiting amino acid should give correspondingly different responses in growth within the limits set by the second limiting amino acid in the basal diet. This graded response in growth is measured quantitatively for known additions of cystine to a given basal

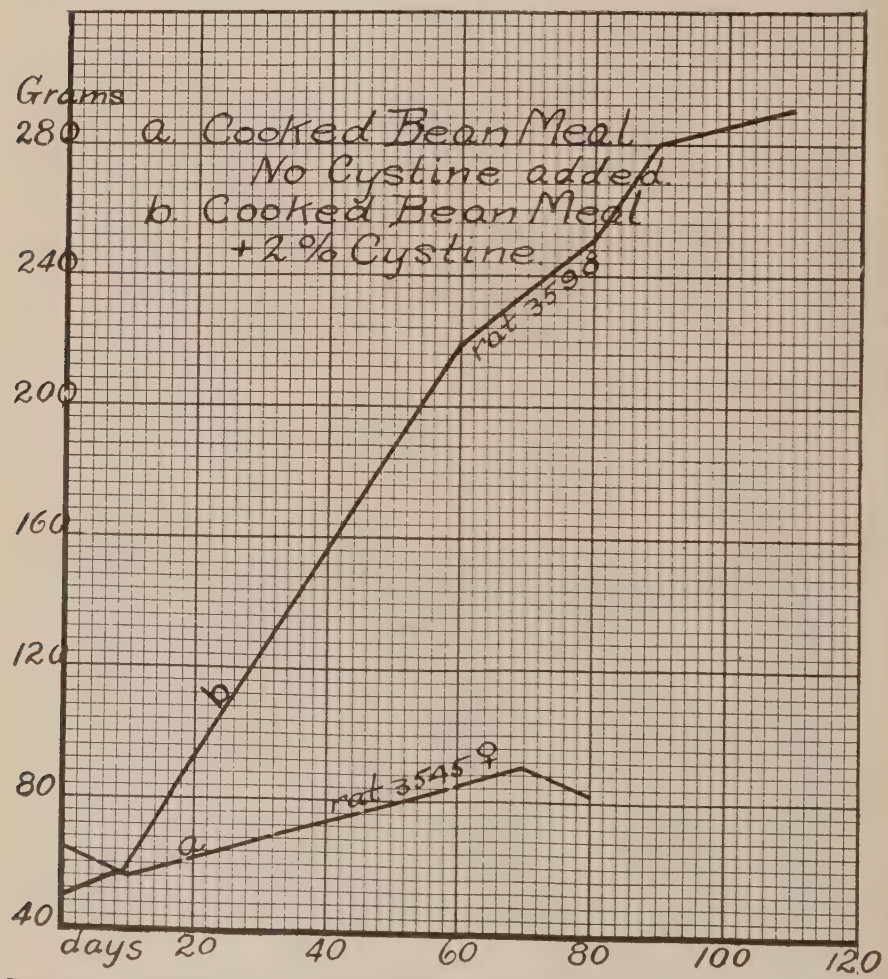


FIG. 250.—CURVE SHOWING INFLUENCE OF A DEFICIENCY OF CYSTINE IN THE DIET. (After Johns and Finks: *Journal of Biological Chemistry*, 41, 379, 1920.)

diet, making it possible to use the method in estimating the amount of that amino acid supplied by (contained in) any given protein which added in known amounts to the same basal diet gives a like increase in the rate of growth.

Procedure.—Compare the gains in weight during an experimental period of 6 weeks, made by standard albino rats (initial age 28–29 days) on a basal diet of limited cystine content but adequate for normal growth in all other respects,

with the gains made by strictly comparable animals on the same diet supplemented by known additions of pure cystine (or by known additions of casein, whose cystine content is to be thus determined).

The basal diet has the following composition:

	Per Cent
Whole milk powder	16.70
Cod liver oil	1.00
Dried brewers' yeast	1.65
Salt mixture[1]	1.65
Starch	79.00

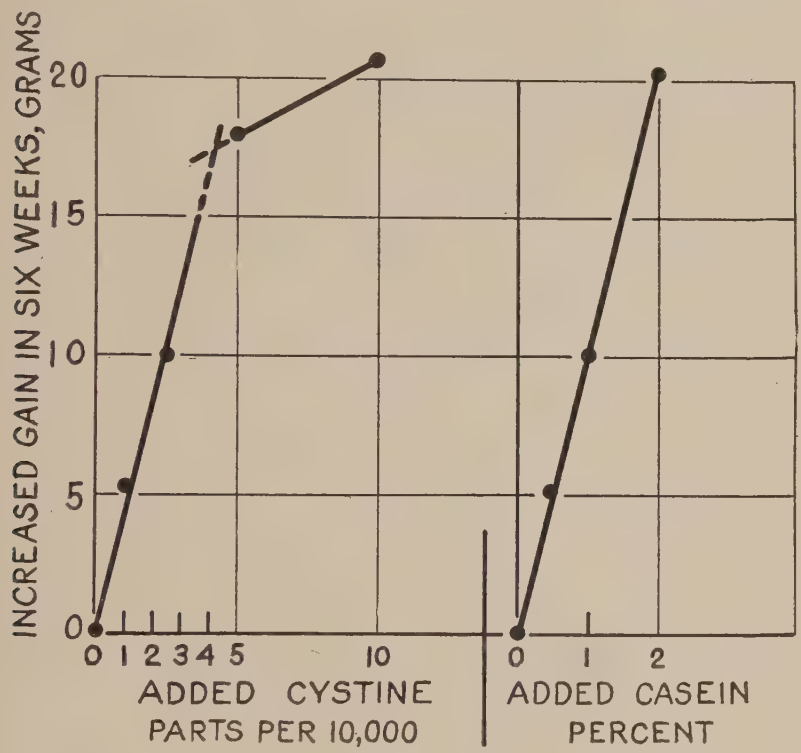


FIG. 260.—QUANTITATIVE RELATIONSHIP OF CYSTINE TO GROWTH.

Rate of gain in weight as related to quantitatively graded additions of cystine or of casein to the same basal diet. In these experiments casein appeared to furnish one-fortieth of its weight of cystine. The region in which cystine is the limiting factor and response in growth is directly proportional to cystine added, is established to 0.025 per cent added cystine with 10 gm. added gain, and appears to extend to an addition of 0.04 per cent of cystine or to an increased gain of about 17 gm. in an experimental period of six weeks. (*Sherman and Woods: Jour. Biol. Chem.*, 66, 29, 1925.)

When casein is introduced into this diet, it replaces an equal amount of the starch. Cystine may be added to the basal diet in graded proportions, e.g., 0.01, 0.025, 0.05, 0.10 and 0.20 per cent.

[1] Osborne and Mendel: *Jour. Biol. Chem.*, 37, 572, 1919. See p. 831.

"When carefully standardized young rats are fed this basal diet, with and without graded additions of cystine, it is possible to determine a direct linear or arithmetical relationship between the added cystine fed and the added growth thereby induced. See Fig. 260.

"Within the range of growth increase in which the response to added cystine was in arithmetical proportion to its amount, it is believed that the response to the added casein may be interpreted as a measure of the cystine (or cystine plus nutritionally equivalent sulfur-containing radicles) furnished by the casein.

"Thus interpreted, these results would indicate that casein contains not less than 1.3 per cent nor more than 2.5 per cent of cystine, or that approximately three-eighths to three-fourths of the total sulfur in casein is in the form of cystine (or of cystine plus other sulfur-containing radicles which are interchangeable with cystine in nutrition)."

(c) Demonstration of Lysine Deficiency.—Make this demonstration the same as (a) above, using the diets listed in the following table:

LYSINE DEFICIENCY DIET

	Diet 1 per cent	Diet 2 per cent
Rolled oats[1]	60.00	60.00
Gelatin[1]	0.00	10.00
Dextrin or starch	30.30	20.30
Salt mixture	4.70	4.70
Butter fat	5.00	5.00

The rat receiving Diet 2 will grow normally because of the high lysine content of gelatin. The animal receiving Diet 1 will fail to grow properly because of lysine deficiency. See Figs 261, 262, and 263.

3. Influence of Carbohydrate Deficiency.—Carbohydrates occupy a very prominent place in the diet of man. That they are not *essential* dietary constituents, at least for the white rat, may be shown by the following experiment.[2]

Demonstration on Carbohydrate Deficiency.—Use young white rats as subjects and proceed as in Experiment (a), p. 839 feeding one rat Diet 1 and another rat Diet 2 as given in the following table:

[1] Oat proteins are low in lysine. Gelatin is relatively high in this amino acid (see p. 118).

[2] Osborne, Thomas B., and Mendel, Lafayette B.: *Soc. Exp. Biol. and Med.*, 18, 136, 1921.

CARBOHYDRATE DEFICIENCY DIET

	Diet 1 Per cent	Diet 2 Per cent
Casein	55.00	20.00
Butter fat	30.00	15.00
Lard	15.00	10.00
Starch or dextrin	0.00	55.00
Yeast, dried,[1] gram per day	0.5	0.5

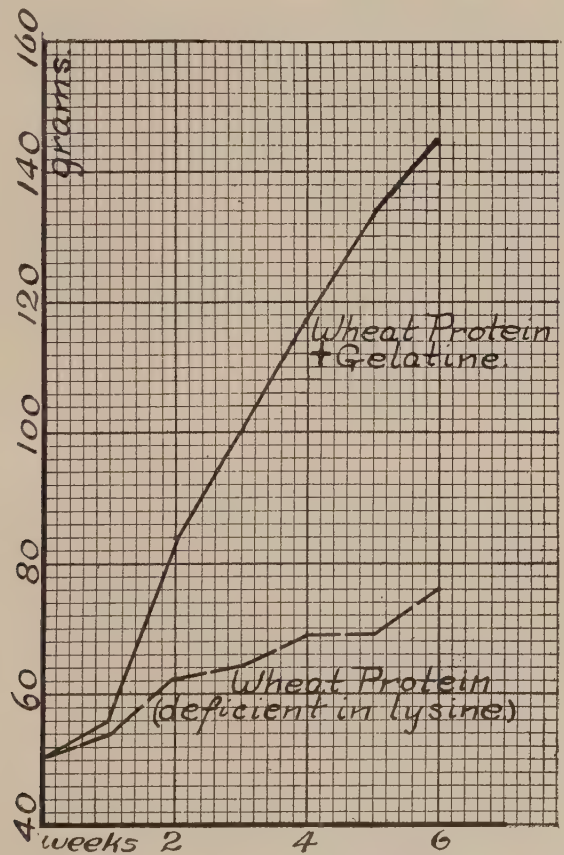


FIG. 261.—CURVE SHOWING INFLUENCE OF A DEFICIENCY OF LYSINE IN THE DIET. (Hawk, Smith and Bergiem: Unpublished data.)

Both rats will grow normally in spite of the practical absence of carbohydrates in Diet 1. In the case of man, the withdrawal of carbohydrates is followed by ketosis (see p. 872). This ketosis is absent or much less pronounced in the case of the white rat.

[1] The yeast is fed separately 0.5 gram to each rat. It may be fed as pills made from moistened yeast. The only carbohydrate present in Diet 1 is the very small amount in the dried yeast.

4. Influence of Fat Deficiency.—That fats are not essential dietary constituents provided we derive sufficient energy from carbohydrates and proteins and a proper supply of "Fat-soluble A" from some suitable non-fat source is easy of demonstration.[1]

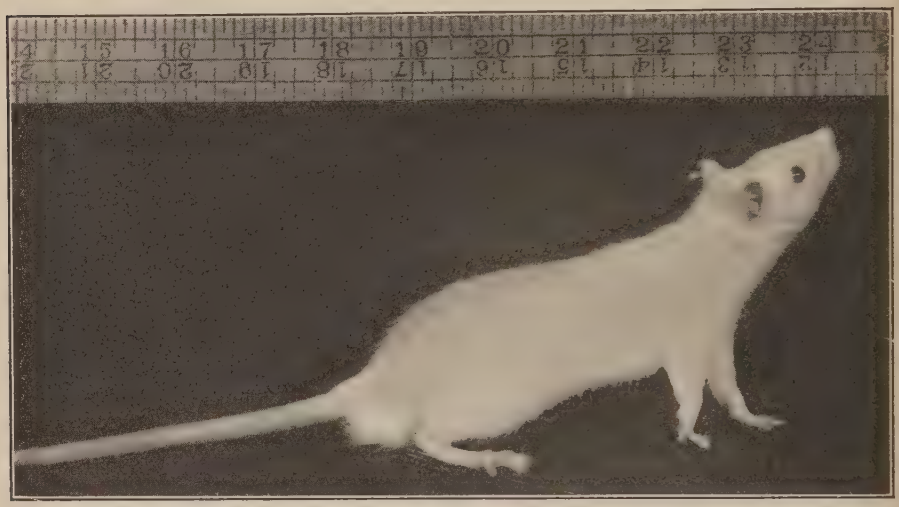


FIG. 262.—RAT RECEIVING WHEAT PROTEIN AND GELATIN. THIS DIET CONTAINED SUFFICIENT *Lysine*. COMPARE FIG. 263.
(Hawk, Bergeim and Smith: Unpublished data.)

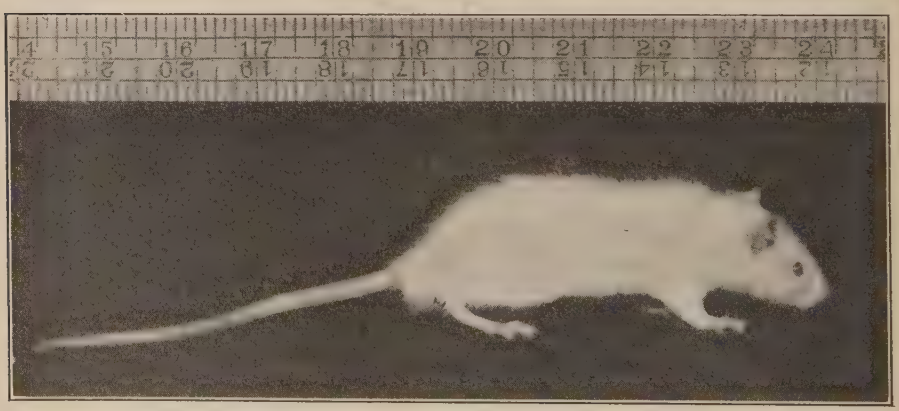


FIG. 263.—RAT RECEIVING WHEAT PROTEIN ONLY. THIS DIET WAS DEFICIENT IN *Lysine*. COMPARE FIG. 262.

Demonstration on Fat Deficiency.—Place two young white rats (30–60 grams) in individual cages. Feed one the adequate diet listed as Diet 1 in the following table. Feed the other rat Diet 2, in which alfalfa or spinach is substituted for the butter fat.

[1] Osborne, Thomas B., and Mendel, Lafayette B.: *Jour. Biol. Chem.*, 45, 145, 1920.

FAT DEFICIENCY DIET

	Diet 1 Per cent	Diet 2 Per cent
Casein	20.0	20.0
Starch	56.0	56.0
Salt mixture	4.0	4.0
Yeast	5.0	5.0
Butter fat	15.0	0.0
Dried alfalfa or spinach	0.0	15.0

Both rats will grow normally, indicating that a fat-free diet is satisfactory for growth, provided the diet in question contains ample "Fat-Soluble A" vitamin. Hindhede[1] claims that fat is not an essential dietary constituent provided sufficient fruit and vegetables are eaten to supply vitamins.

5. Influence of Energy Deficiency.—The bulk of the energy in the accustomed diet of man is furnished by carbohydrates and fats. Therefore, if we reduce the dietary content of these substances to the minimum and maintain other dietary factors (vitamins, protein, salts, water, etc.) at a normal level, we will not be properly nourished. The body will attempt to derive the necessary energy by the combustion of body tissues and a pronounced and rapid loss in body weight accompanied by other signs of abnormality will soon follow.

Demonstration on Energy Deficiency.—(a) Demonstration on man, following the suggestions embraced in the preceding paragraph.

(b) Demonstration on the White Rat.—Place two young white rats (100–150 grams) in individual cages. Feed one the adequate diet listed as Diet 1 below. Feed the second animal the same diet minus the carbohydrate. This animal will lose weight rapidly, due to the low energy value of the diet whereas the other rat will grow normally.

ENERGY DEFICIENCY DIET

	Diet 1 Grams per day	Diet 2 Grams per day
Casein	2.0	2.0
Starch	6.0	0.0
Salt mixture	0.4	0.4
Yeast (dry)	0.5	0.5
Butter fat	1.5	0 8
Alfalfa or spinach (dry)	0.0	0.7

6. Influence of Inorganic Matter Deficiency.—A demonstration of the harmful effect following the elimination of calcium from the diet may readily be made if the diets listed below be fed to young white rats.

[1] Hindhede: *Skand. Arch. Physiol.*, 39, 78, 1920.

DEFICIENCY OF CALCIUM DIET

	Diet 1 Per cent	Diet 2 Per cent
Beef liver (steamed and dried)[1]	20.0	20.0
Casein	10.0	10.0
NaCl	1.0	1.0
KCl	1.0	1.0
$CaCO_3$	0.0	1.5
Dextrin or starch	65.0	63.5
Butter fat	3.0	3.0

(a) **Demonstration on Calcium Deficiency.—Place two young white rats (40–60 grams) in separate cages and feed the diets listed above. Make frequent**

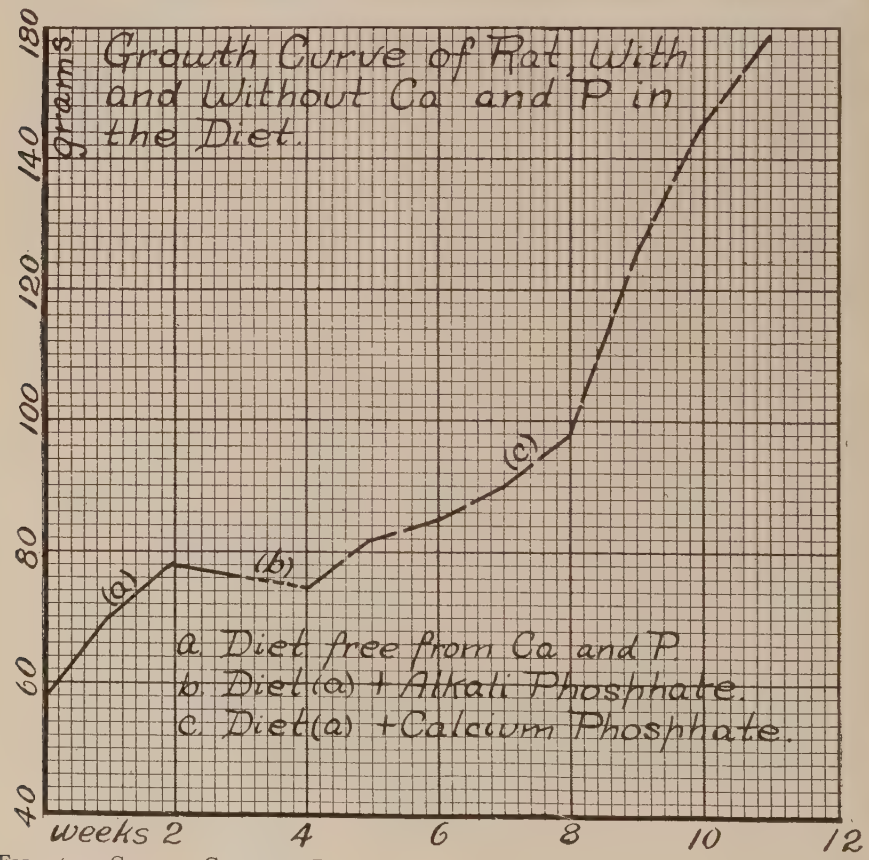


FIG. 264.—GROWTH CURVE OF RAT WITH AND WITHOUT CALCIUM AND PHOSPHORUS IN THE DIET.
(Bergeim, Smith and Hawk: Unpublished data.)

body weight determinations. The rat receiving Diet 2 will show normal growth. The rat receiving Diet 1 will fail to show normal gains in weight. This diet is deficient in calcium. See Fig. 264 and 265.

[1] Beef liver contains Water-Soluble B and Fat-Soluble A. The diet is adequate except for calcium (McCollum, Simmons, Parsons, Shipley, and Park; *Jour. Biol. Chem.*, 45, 333, 1921).

(b) Influence of Ultraviolet Light on Inorganic Metabolism.—For demonstration, see Experiment 1, page 829. Ultraviolet radiation has calcium-depositing properties.

(c) Iron Deficiency.—The necessity for *iron* in the normal dietary has received particular emphasis in its relation to anemia.[1] A diet rich in "complete" proteins and *iron*—particularly liver—has been found efficient in the treatment of pernicious anemia. The belief of Abderhalden that hemoglobin can be formed from inorganic iron in the diet only when there is associated with it certain organic groups (pyrrole?) such as are contained in chlorophyll, has been confirmed by the experiments of Hart, Steenbock, Elvehjem and Waddell.[2]

FIG. 265.—SKELETONS OF TWIN ALBINO RATS SHOWING INFLUENCE OF CALCIUM DEFICIENCY. (*Sherman and MacLeod: Jour. Biol. Chem.*, 64, 429, 1925.)

(d) Iodine Deficiency.—Iodine is essential for the formation of thyroxin (see p. 481), the hormone of the thyroid gland, which regulates metabolism. In the absence of iodine from the diet, a condition of hyperplasia of the thyroid gland (goiter) results, which can be cured or prevented by the addition of this element to the diet. It is important to note, however, that goiters may be due to causes other than iodine insufficiency.[3] It is for this reason that the indiscriminate administration of iodine should be discouraged. A hyperfunctioning thyroid gland may moreover be aggravated by too liberal use of iodine.

7. Influence of Water Deficiency.—(a) The importance of water in nutrition may be shown very satisfactorily on guinea pigs. Proceed as follows: Place two young guinea pigs (150–200 grams) in separate cages, and give each free

[1] Minot and Murphy: *Jour. Am. Med. Assn.*, 87, 470, 1926.
Editorial: *Jour. Am. Med. Assn.*, 87, 672, 1926.
McGowan and Crichton, *Bioch. Jour.*, 17, 204, 1923; 18, 265 and 273, 1924.
[2] Hart, Steenbock, Elvehjem and Waddell: *J ur. Biol. Chem.*, 65, 67, 1925.
[3] Endocrinology and Metabolism, Vol. 1, New York, Appleton and Co., 1924.

access to a diet of hay, oats, and lemon or orange juice which has been dried rapidly at a low temperature. Permit one pig water ad lib., and give the second pig no water. The pig receiving water will remain normal and will exhibit normal gain in body weight. The pig receiving no water will soon show pronounced losses in body weight and other signs of abnormality. The animal will die in a short time unless water is added to the diet. This experiment demonstrates very clearly that water is an indispensable dietary constituent. In fact, watar is more important than food. The following experiment will show this:

(b) Food Starvation vs. Water Starvation.—Place two young guinea pigs (150–200 grams) in separate cages. Give one a diet such as that described on page 833 (c), but permit the animal no water. Give the second pig no food, but permit free access to water. The pig receiving no water will quickly become abnormal, and it will be necessary to give it water to preserve its life. The second pig, which has access to water[1] but receives no food to eat, will live longer than the pig receiving an abundance of dry food. This little experiment impresses the important fact that man can live longer without food than without water. By restricting the amount of water in the diet of an albino rat, the animal may be kept at constant body weight for several weeks although the diet is otherwise adequate. In many respects the effect is similar to that of underfeeding. Some increased tolerance to water restriction may develop during such a test.[2]

8. Adequate vs. Optimal Nutrition. The Dietary Efficiency of Milk.[3]—The foregoing experiments have demonstrated that certain factors must be present in a diet if it is to be deemed *adequate*. But such a diet although it qualifies under all required nutritional standards may be improved upon. We thus form what may be termed an *optimal* diet. To demonstrate the difference between an *adequate* and an *optimal* diet proceed as follows:

Place two white rats from the same litter, each weighing 35–45 grams, in separate cages. Feed one rat an *adequate* diet (see below) and the other an *optimal* diet (see below).

Adequate Diet		*Optimal Diet*	
Whole wheat	5 parts	Whole wheat	2 parts
Whole milk (powder)	1 part	Whole milk (powder)	1 part

It will be noted that dry milk constitutes only *one-sixth* of the adequate diet whereas it makes up *one-third* of the optimal diet. Give the animals water *ad lib.* and weigh them at least twice a week. Continue the experiment for at least ten weeks, plotting the growth curve of each animal (see Fig. 242) and keeping an accurate record of the food eaten (see Fig. 240). If it is desired to investigate the relationship of these diets to reproduction, two rats of opposite sex may be used in each test and caged together).

[1] In case the pig does not drink the water, the animal should be fed the fluid by a sound.
[2] Kudo: *Jour. Exptl. Zool.*, 33, 435, 1021.
[3] Sherman: Chemistry of Food and Nutrition, Third Edition, page 561, Macmillan New York, 1926.

The rat (or rats) receiving the *optimal* diet should grow more rapidly than the animal or animals receiving the *adequate* diet. If the experiment embraces the question of reproduction it will be found that the rats on the optimal diet have greater success in the rearing of young and that the offspring grow better during the nursing period than do the offspring of the rats ingesting the *adequate* diet.

In a comprehensive series of observations on these diets by Sherman and associates it has been found that the animals fed the optimal diet mature earlier and live longer. In common with other data secured from tests with albino rats these findings may be applied directly to the human dietary, and afford a very striking demonstration of *the remarkable nutritive efficiency of milk.*

III. EXPERIMENTS ON NITROGEN METABOLISM

1. Time Relations of Protein Metabolism.—It is a well-known physiological fact that an interval elapses between the ingestion of protein food and the appearance in the urine of certain products representing the complete catabolism of this food. For example, if one ingests an excess of protein material an interval elapses before the urine gives evidence of the complete excretion of certain products representative of the catabolism of the protein. Urea is the chief of these. The term "nitrogen lag" has been used to designate the period elapsing between the ingestion of protein and the excretion in the urine of a quantity of nitrogen equivalent to that contained in the protein.

Experiment.—Ingest a simple uniform diet whose exact composition has been determined by analysis or whose approximate composition has been estimated. (See table page 850.) Continue this diet from one to four days. Collect the urine in two-hour periods from 7 A. M. to 11 P. M. and in an eight-hour period between 11 P. M. and 7 A. M. Analyze each specimen for total nitrogen or urea. At the end of this preliminary period add to the uniform diet, at one meal, a weighed quantity (150–250 grams) of lean meat specially prepared and analyzed. Collect the urine in periods as before and determine total nitrogen or urea. Calculate the total nitrogen or urea excretion; tabulate the data and plot curves showing the course of the nitrogen excretion on the various days of the experiment. How long was the "nitrogen lag?"

A less accurate experiment than the above but one which yields interesting data may be carried out as follows:

Ingest a simple diet whose nitrogen content can be estimated with some degree of accuracy (see table p. 850). Collect the urine in two-hour periods from 7 A. M. to 11 P. M. and in an eight-hour period from 11 P. M. to 7 A. M. and analyze for total nitrogen or urea. The next day ingest the same diet plus 150–250 grams of lean meat whose nitrogen content has been determined by

analysis or estimated. Collect the urine as upon the previous day and determine its total nitrogen or urea content. Plot curves showing the course of the nitrogen or urea excretion on each of the days. How soon after the ingestion of the large quantity of meat did you note an increase in the nitrogen or urea excretion? How many hours after the meal was the maximum quantity of nitrogen or urea excreted?

COMPOSITION OF COMMON FOODS[1]

Food		Water	Protein (N×6.25)	Fat	Carbohydrate	Ash	Calories per pound
		Per cent	Per cent	Per cent	Per cent	Per cent	
Beef	Loin	61.3	19.0	19.1	.....	1.0	1125
	Ribs	57.0	17.8	24.6	.....	0.9	1338
	Round	67.8	20.9	10.6	.....	1.1	812
Mutton	Loin	50.2	16.0	33.1	.....	0.8	1642
	Leg	67.4	19.8	12.4	.....	1.1	865
	Shoulder	67.2	19.5	12.9	.....	1.0	905
Sweetbreads		70.9	16.8	12.1	.....	1.6	800
Liver		65.6	20.2	3.1	2.5	1.3	539
Heart		62.6	16.0	20.4	.....	1.0	1125
Tongue		70.8	18.9	9.2	.....	1.0	719
Brain		80.6	8.8	9.3	.....	1.1	540
Ham		49.2	22.5	21.0	.....	5.8	1266
Chicken	Broilers	74.8	21.5	2.5	.....	1.1	492
	Fowl	63.7	19.3	16.3	.....	1.0	1016
Fish	Halibut	75.4	18.6	5.2	.....	1.0	550
	Salmon	64.6	22.0	12.8	.....	1.4	922
Cereals	Oatmeal (boiled)	84.5	2.8	0.5	11.5	0.7	280
	Rice (boiled)	72.5	2.8	0.1	24.4	0.2	498
	Shredded Wheat	8.1	10.5	1.4	77.9[2]	2.1	1660
Crackers	Graham	5.4	10.0	9.4	73.8[2]	1.4	1904
	Oatmeal	6.3	11.8	11.1	69.0[2]	1.8	1920
	Soda	5.9	9.8	9.1	73.1[2]	2.1	1875
Bread	White	35.3	9.2	1.3	53.1[2]	1.1	1182
	Graham	35.7	8.9	1.8	52.1[2]	1.5	1189
Potatoes	Boiled	75.5	2.5	0.1	20.9[2]	1.0	429
	Mashed	75.1	2.6	3.0	17.8	1.5	493
	Chips	2.2	6.8	39.8	46.7	4.5	2598
	Sweet	51.9	3.0	2.1	42.1	0.9	903
Eggs (hen)	White	86.2	12.3	0.2	.....	0.6	231
	Yolk	49.5	15.7	33.3	.....	1.1	1643
	Entire edible portion	73.7	13.4	10.5	.....	1.0	672
Milk		87.0	3.3	4.0	5.0	0.7	314
Butter		12.7	1.3	84.0	.....	1.9[3]	3450
Peanut butter		2.1	29.3	46.5	17.1	5.0	2741
Soups	Consommé	96.0	2.5	.....	0.4	1.1	53
	Tomato	90.0	1.8	1.1	5.6	1.5	179
	Pea	86.9	3.6	0.7	7.6	1.2	232
	Celery (cream)	88.6	2.1	2.8	5.0	1.5	243
Tapioca pudding		64.5	3.3	3.2	28.2	0.8	702
Doughnuts		18.3	6.7	21.0	53.1[2]	0.9	1942
Ginger snaps		6.3	6.5	8.6	76.0[2]	2.6	1848
Peas (cooked)		73.8	6.7	3.4	14.6	1.5	525
Lettuce		94.7	1.2	0.3	2.9[2]	0.9	87
Apples		84.6	0.4	0.5	14.2[2]	0.3	285
Oranges		86.9	0.8	0.2	11.6	0.5	233
Bananas		75.3	1.3	0.6	22.0[2]	0.8	447
Figs		79.1	1.5	.....	18.8	0.6	368
Sandwiches	Egg	41.4	9.6	12.7	34.5	1.8	1319
	Chicken	48.5	12.3	5.4	32.1	1.7	1026
Cheese (American)		30.0	28.8	35.9	0.3	5.0[4]	1990

[1] Sherman's "Food Products," Macmillan, 1925.

[2] Percentage of fiber, included under carbohydrate: shredded wheat (1.7), Graham crackers (1.5), oatmeal crackers (1.9), soda crackers (0.3), white bread (0.5), Graham bread (1.1), boiled potatoes (0.6), doughnuts (0.7), ginger snaps (0.7), lettuce (0.7), apples (1.2), bananas (1.0).

[3] Including salt.

[4] Including salt and sugar.

2. **Protein Utilization.**—By "protein utilization" is meant the percentage of the ingested protein which is actually absorbed and assimilated.

This may be determined by the following procedure: Ingest any diet of known nitrogen content for a period of three days[1] (see table, page 850). Collect all feces from the diet making the "separations" as directed on page 791, using carmine as the initial "marker" and charcoal as the final "marker" or vice versa Preserve the feces as directed on page 790. Mix the total feces thoroughly and determine the nitrogen by the Kjeldahl method (see Chapter XXXI and note on page 546). The approximate nitrogen utilization may be calculated as follows:

$$\frac{(\text{Food nitrogen} - \text{Feces nitrogen}) \times 100}{\text{Food nitrogen}} = \text{Approximate percentage nitrogen utilization.}$$

If it is desired to ascertain the actual percentage of the ingested nitrogen which has been utilized by the body we must make a correction for metabolic nitrogen. In doing this proceed as follows: Ingest a non-nitrogenous diet as described on page 862 for a period of two days, using sufficient agar-agar to insure a daily fecal output which shall approximate in weight that obtained when the regular protein diet was ingested.[2] Separate and preserve the feces as directed on page 790. Mix thoroughly and analyze for nitrogen according to the Kjeldahl method (see Chapter XXXI and note on page 546). Calculate the actual percentage utilization of the diet as follows:

$$\frac{[\text{Food nitrogen} - (\text{Fecal nitrogen} - \text{Metabolic nitrogen})] \times 100}{\text{Food nitrogen}} = \text{Actual percentage nitrogen utilization.}$$

If urinary nitrogen is determined the above data enable us to prepare a nitrogen balance (see Experiment 6, page 870).

3. **Protein-Sparing Action of Carbohydrate and Fat.**—The non-nitrogenous nutrients, carbohydrate and fat, have the power to diminish the extent of the catabolism of protein in the normal human body. In other words they are said to "spare" protein. This point is illustrated in data reported by von Noorden and Dieters, which are tabulated below.

PROTEIN-SPARING ACTION OF CARBOHYDRATE AND FAT

Nitrogen ingested	Nitrogen in urine	Remarks
12.6 grams.	10.4 grams.	
12.6 grams+200 grams sucrose.	9.0 grams.	13 per cent reduction in protein catabolism.

It will be observed that the addition of 200 grams of sucrose to the diet was accompanied by a decrease of 13 per cent in the amount of protein catabolized.

[1] See note 1, p. 792.

[2] It is frequently difficult to so regulate the agar-agar intake as to secure the proper fecal output. In such an event the proper value for metabolic nitrogen must be obtained by calculation. For example if 89.1 grams of feces were excreted per day on the protein diet, and 166.5 grams per day (with a nitrogen value of 0.5 gram) when agar was employed, the actual value for metabolic product nitrogen may be obtained by the following proportion, assuming that the content of metabolic nitrogen is proportional to the weight of feces excreted: $89.1 : 166.5 :: x : 0.5$. $x = 0.268$ gram metabolic nitrogen per day.

It has been established that carbohydrates are more efficient "protein sparers" than are the fats. For example Voit found carbohydrate to produce a 9 per cent decrease in protein catabolism whereas fats produced only a 7 per cent decrease.

Experiment.—Ingest a uniform diet of known or estimated nitrogen content for a period of four days. Collect and preserve the urine accurately (see page 789) in 24-hour samples and analyze the excretion of the *third and fourth days* for total nitrogen. On the fifth day *add 200 grams of sucrose to the diet.* Analyze this urine also for total nitrogen. Calculate your results and tabulate the data as shown in table on page 851.

Did the sucrose influence the catabolism of protein in your body?

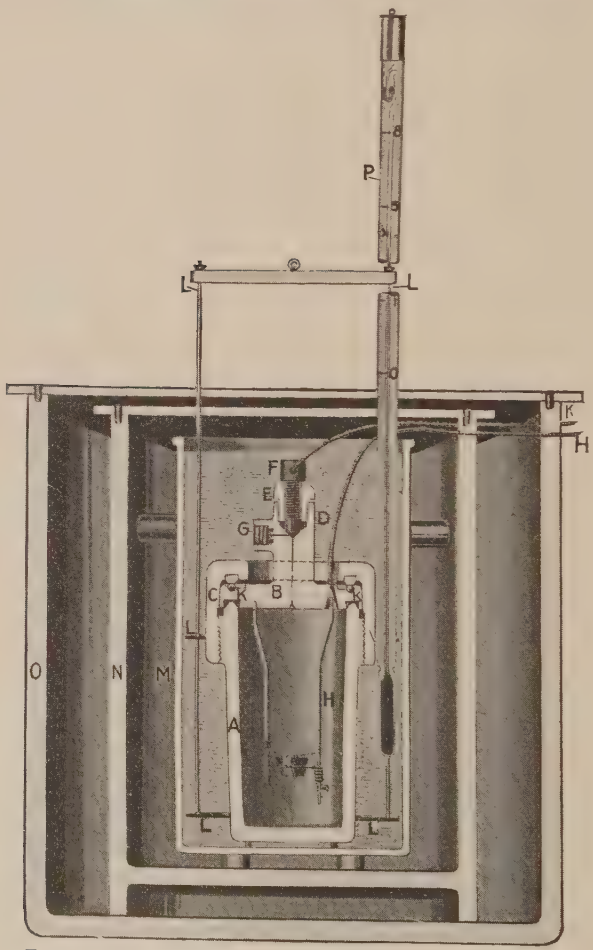


FIG. 266.—BERTHELOT-ATWATER BOMB CALORIMETER.

4. **Influence of a High Calorie Non-nitrogenous Diet.**—If an individual fasts there is a combustion of a certain amount of protein tissue each day of the fast. The destruction of such tissue is rather low on the first day due to the fact that the glycogen stores of the body are being utilized to furnish the necessary energy. If an individual

instead of fasting, ingests a diet of high calorific value and very low in nitrogen the output of nitrogen in the urine of the third or fourth day will be less than on the third or fourth day in fasting. This is due to the fact that the body derives sufficient energy from the high calorie diet and there is less destruction of protein body tissues than occurs in fasting. For a discussion of energy value of foods see "Determination of Fuel Value of Foods," below, and the table on page 850.

Experiment.—Ingest a high calorie diet which is very low in nitrogen or actually non-nitrogenous. A satisfactory diet may include sugar, butter, starch, cream, agar-agar and water. (For energy values see below and table, page 850.) Ingest such a diet for three days. Collect the urine in 24-hour periods, preserve and analyze it for total nitrogen, acidity and ammonia. Note the low nitrogen excretion on the third day as compared with the nitrogen output of the third day of fasting. If so desired, you may (at some later date) fast for three days and repeat the above analyses for comparison.

Determination of Fuel Value of Food.—When organic substances are oxidized or burned in the human body they liberate a certain amount of heat. This calorific energy or heat value varies according to the type of organic matter undergoing oxidation. Thus the proteins, fats and carbohydrates of the diet when they are burned in the body yield different quantities of heat per unit of substance than do organic acids, alcohol, etc. The energy values of pure protein fat and carbohydrate are the following:

Protein	= 4.1 large calories per gram.
Fat	= 9.3 large calories per gram.
Carbohydrate	= 4.1 large calories per gram.

In arriving at the energy value of any given diet it is customary to burn weighed samples of the various foods in an oxygen atmosphere in an apparatus called a bomb calorimeter (see Fig. 266, page 852). By this means we may determine how much heat is liberated when the ingested food is oxidized in the body. A correction must be made for the incompletely oxidized substances of the urine and feces. A large mass of data concerning the heat value of foods has been collected and tabulated, and it is therefore possible to arrive at an approximate idea of the energy value of a diet by calculation (see table, page 850). The bomb calorimeter shown in Fig. 266, page 852, is one of the most satisfactory for actual determination of the heat of combustion of organic substances.

The Oxycalorimeter of Benedict and Fox.—The complexity of manipulation and calculation and the high initial cost of the bomb calorimeter are absent in the "oxy-calorimeter" an apparatus developed in the Nutrition laboratory of the Carnegie Institution of Washington, Boston.[1] The principle involved in the oxy-calorimeter is the direct determination of the volume of oxygen required to burn a known weight of a substance, and the computation therefrom of the potential energy of the substance, based upon a series of factors for the calorific value of a liter of oxygen (p. 854) previously established by combustion of similar material in a bomb calorimeter. The gaseous and energy relationship in the combustion of some commonly metabolized compounds are shown in the following table:

[1] Benedict and Fox: *Ind. and Eng. Chem.*, 17, 912, 1925; *Jour. Biol. Chem.*, 66, 783, 1925.

Substance	Oxygen required to oxidize 1 gm., c.c.	Produced in the oxidation of 1 gm.		Calories per liter of oxygen
		Carbon dioxide, c.c.	Heat, cals.	
Starch	829.3	829.3	4.20	5.06
Cane sugar	785.5	785.5	3.96	5.04
Glucose	746.2	746.2	3.74	5.01
Lactic acid	745.9	746.0	3.62	4.85
Animal fat	2013.2	1431.1	9.50	4.72
Human fat	1990.8	1420.4	9.54	4.79
Protein	956.9	773.8	4.40	4.60
Acetone	1542.9	1157.2	7.43	4.82
β-Hydroxybutyric acid	968.2	860.7	4.69	4.85
Ethyl alcohol	1459.5	972.9	7.08	4.85

It will be seen that the range of calorific value *per gm. of foodstuff* is wide (column 4), while the calorific value *per liter of* O_2 (column 5) varies only between 4.6 and 5.1.

This procedure requires a combustion chamber, a method of ignition, and the control of the combustion, in order to insure complete conversion of all the carbon and hydrogen in the sample to carbon dioxide and water and the liberation of nitrogen in the free form in the case of nitrogenous materials. A known weight of dried substance is burned in a confined volume of nearly pure oxygen, with provision for the rapid absorption by soda-lime of the carbon dioxide produced and the return of the purified oxygen to the combustion chamber. The contraction in volume due to the absorption of oxygen in the process of combustion may be measured by a small expansion chamber, the top of which is brought back to its original level by introducing a measured volume of air or oxygen after the combustion ceases (see student respiration apparatus, Fig. 155), or it may be measured by noting quantitatively on a calibrated spirometer bell the decrease in the apparent volume of air in the entire apparatus (Fig. 267).

Any one of the numerous simple forms of closed circuit respiration apparatus may readily be adapted to the direct determination of the oxygen consumption.

When the respiration apparatus is used as an oxy-calorimeter, the usual inspiratory and expiratory valves are removed and the apparatus is connected with a combustion chamber, *A*, and a motorblower unit, *C*.

A typical calculation of the heat of combustion of a sample of mixed food (salmon salad sandwich) is as follows:

The volume of O_2, corrected for temperature and pressure, required to burn 1 gm. of the dried substance was 941 c.c.

The nitrogen in the sample was found to be 3.1 per cent by weight, or 25 c.c. per gm. of substance. This volume of nitrogen took the place of oxygen absorbed and should therefore be added to the amount of oxygen measured per gm.; *i.e.*, 941 + 25 = 966 c.c., which represents the volume of oxygen required to burn completely 1 gm. of the salmon salad sandwich.

Although no proximate analysis of the material was made, one can readily assume that it was made up in large part of carbohydrate and hence the calorific value of oxygen would probably be somewhat less than that for pure starch, *i.e.*,

5.06. According to the combustion in the bomb calorimeter, each gm. of the sample of salmon salad sandwich had an energy value of 4826 calories. The calorific equivalent of 1 liter of oxygen in the case of this material therefore is 5.00 calories (4826 ÷ 966).

Tables listing the calorific values of oxygen for some commonly metabolized compounds and for a number of various foods and excreta are given in the original papers. Average calorific values of oxygen for use in connection with determina-

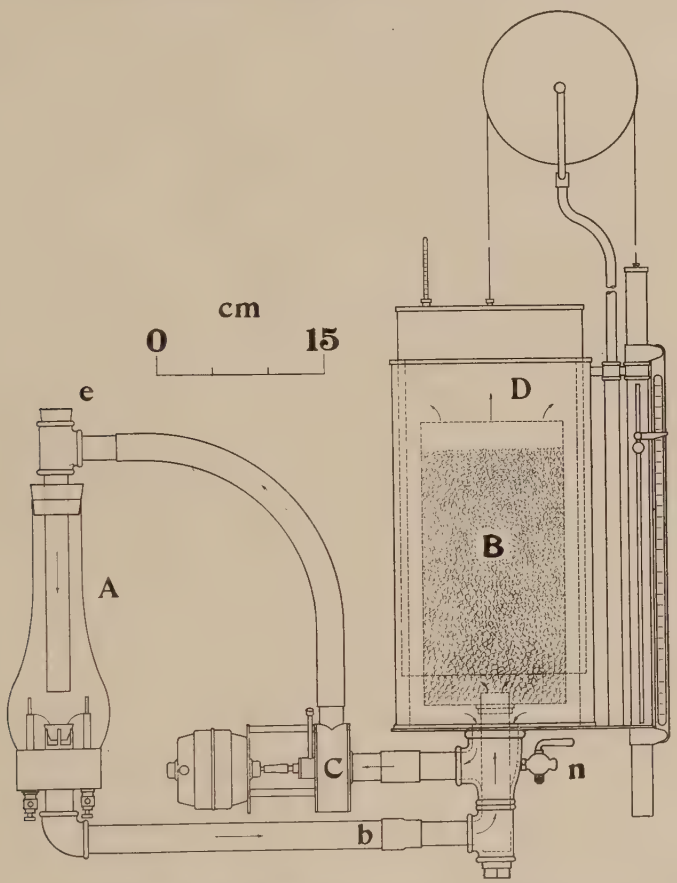


FIG. 267.—OXYCALORIMETER OF BENEDICT AND FOX. (*Benedict and Fox: Jour. Biol. Chem.*, 66, 783, 1925.) See Fig. 155.

tions with the oxy-calorimeter are suggested as follows: for nitrogen-rich substances 4.68 calories per liter of oxygen; for fats 4.7 calories; for carbohydrate-rich foods 5.0 calories; for a mixed diet of carbohydrates and fats 4.825 calories; and for feces 5.0 calories.

5. Influence of Purine-free and High Purine Diets.—The uric acid of the body has a two-fold origin, *i.e.*, it may arise from the metabolism of the purine (nuclein) material of body tissue (glandular organs in particular) or it may arise from the ingestion of purine (nuclein)

material. That uric acid which arises from the first source is called *endogenous* while that which arises from the second source is termed *exogenous*. Secretory activity may also act to increase the endogenous uric acid output. The urine will therefore contain uric acid even though no precursor of the acid be ingested. We may also increase the uric acid output markedly by ingesting a high purine diet. However, no matter how much purine material is eaten, only a small part of this purine material reappears in the urine as uric acid. In gout it is claimed there is an accumulation of uric acid in the blood due to the fact that the kidney has lost the ability to maintain the normal blood uric acid level. In this disease the excretion of uric acid may be low before an attack and increase considerably during an attack. The excretion of exogenous uric acid in gout is also much slower than normal.

Experiment.—Ingest a purine-free diet containing about 16 grams of nitrogen and consisting of egg, cheese, milk, starch, fruit, sugar and water for a period of two days (for purine content of foods, see table, page 857). Determine or estimate the nitrogen content (see table, page 850) and during the next two days substitute sweetbreads, thymus or liver for all the nitrogen of the diet maintaining the calorific value of the diet the same as before. Return to the original purine-free diet for a third interval of two days. During the final period of two days feed a diet of sweetbreads or liver containing 50 per cent more nitrogen than that of the first sweetbread period. Collect the urine for each of the eight days of the experiment and determine uric acid, and total nitrogen or urea. Note the rise in the uric acid output during the sweetbread periods. The uric acid output on the purine-free diet is endogenous in origin. Tabulate your results. The data on page 857 were secured by Taylor and Rose[1] in a similar but much more carefully controlled test than that just outlined.

6. **A Study of Endogenous Uric Acid Output.**—The uric acid in the urine is said to have two sources, *i.e.*, from the purine material of the tissues and from the purine material ingested. The former is *endogenous* uric acid, the latter *exogenous* uric acid.[2] The output of uric acid on the purine-free diet in Experiments 15 and 17 is *endogenous*. Mares[3] claims that food-stuffs act to increase the endogenous uric acid output by stimulating the digestive glands to activity. A similar finding is reported by Mendel and Stehle.[4] The food-stuff having the most pronounced influence was protein. *Pilocarpine* which stimulates the digestive glands was found to increase the endogenous uric acid output whereas *atropine* which inhibits secretory activity was found to decrease the output of endogenous uric acid. The influence of protein upon the endogenous uric acid excretion is

[1] Taylor and Rose: *Jour. Biol. Chem.*, 14, 419, 1913.
[2] Burian and Schur: *Zeit. physiol. Chem.*, 43, 532, 1904–5
[3] Mares: *Arch. f. d. ges. Physiol.*, 134, 59, 1910.
[4] Mendel and Stehle: *Jour. Biol. Chem.*, 22, 215, 1915.

INFLUENCE OF PURINE-FREE AND HIGH PURINE DIETS

Nitrogen ingestion 10 grams daily
(Taylor and Rose)

Urinary constituents determined (grams per day)	Purine-free diet	Purine diet (medium)	Purine diet (increased)	Purine-free diet
Uric acid N	0.09	0.14	0.24	0.07
Total nitrogen	8.9	8.7	9.1	8.8
Urea N ($+NH_3$)	7.3	7.1	7.1	7.05
Creatinine	1.57	1.49	1.51	

PURINE CONTENT OF FOODS[1]

(Percentage purine base nitrogen)

Food	Analyzed by			Food	Analyzed by		
	Vogel[2]	Hall[3]	Bessau and Schmid[4]		Vogel[2]	Hall[3]	Bessau and Schmid[4]
Beef	0.059	0.052	0.037	Lettuce	.....	.....	0.003
Liver	0.099	0.110	0.093	Cucumbers	.....	.....	None.
Mutton	.....	.....	0.026	Rye bread	0.014	.....	Trace.
Tongue	.....	.....	0.055	White bread	0.008	None.	None.
Chicken	.....	.....	0.029	Milk	0.0002	0.0002	None.
Thymus	0.398	0.403	0.330	Eggs	None.	None.	None.
Cod fish	0.040	0.023	0.038	Cheese	0.0004	.....	None.
Potatoes	0.001	0.0007	0.009	Rice	0.0004	None.	None.
Pancreas	.....	0.393	.....	Tapioca	.....	.....	None.
Peas	0.016	0.016	0.018	Oatmeal	.....	.....	None.
Spinach	0.022	.....	0.024	Onions	.....	.....	None.
Hominy	0.004	.....	.....	Tomatoes	None.	.....	None.

[1] Other purine-free foods not listed here are *fruits*, *butter*, *cream* and *starch*.
[2] Vogel: *Münch. med. Woch.*, 58, 2433, 1911.
[3] Hall: "The Purine Bodies," Philadelphia, 1904.
[4] Bessau and Schmid: *Therap. Monatsch.*, March, 1910.

shown by the chart in Fig. 268. The fasting output by the same individual is shown, for comparison, in Fig. 269.

Experiment.—Ingest a purine-free diet consisting of milk, egg, fruit, cheese, butter, sugar and bread for one day. Continue the diet for breakfast and luncheon the next day but eat nothing after 12 o'clock noon, until 12 o'clock noon the following day, i.e., the third day of the experiment. At that time ingest 125–150

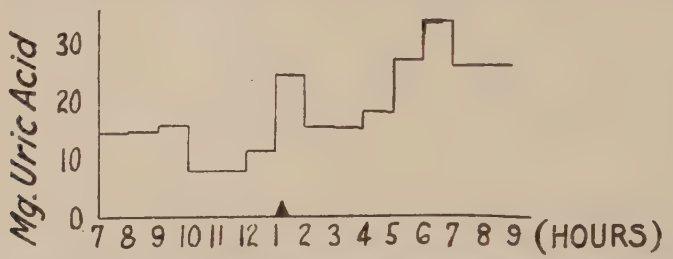


FIG. 268.—INFLUENCE OF PROTEIN INGESTION ON ENDOGENOUS URIC ACID OUTPUT. GLUTEN (130 GRAMS) INGESTED AT 1 P.M. (*Mendel and Stehle: Jour. Biol. Chem.*, 22, 215, 1915.)

grams of gluten or some other purine-free protein preparation. On the fourth day of the experiment eat nothing until 9 P. M.

Collect the urine each day in hour periods from 7 A. M. to 9 P. M. and analyze for uric acid (see methods in Chapter XXXI). Chart your data similarly to those shown in Figs. 268 and 269, and compare them with the findings there recorded.

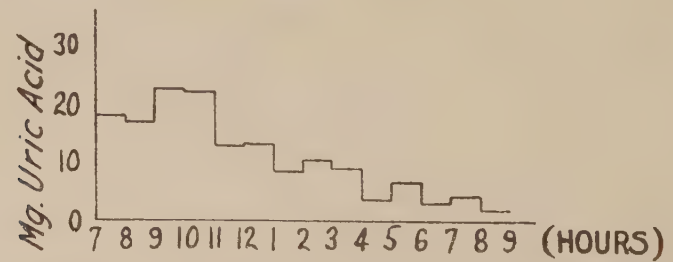


FIG. 269.—THE ENDOGENOUS URIC ACID OUTPUT DURING FASTING. (*Mendel and Stehle: Jour. Biol. Chem.*, 22, 215, 1915.)

7. **The Rate of Purine Excretion.**—The purine material ingested by the average normal person and which is not transformed in the body will be eliminated in about 24 hours. In the case of persons afflicted with gout the purine elimination is delayed. The establishment of this delayed purine elimination is often of diagnostic assistance.

Demonstrate the rate of purine excretion as follows: Ingest a purine-free diet consisting of egg, milk, cheese, starch, sugar, fruit and water for two days and follow this by a day in which sweetbreads, thymus or liver is substituted for one of the meals of the day (see table page 857 for purine content of foods). Finish the experiment by ingesting the original purine-free diet for two days. Collect

each day's urine and analyze for uric acid. How soon after the sweetbread ingestion was the original plane of endogenous uric acid elimination reestablished? If one desires to locate this time more definitely the urine may be collected in short periods (one to two hours) and the uric acid content of each specimen determined. Particularly instructive data may be collected by performing the above experiment on a gout patient and upon a normal person for comparison.

8. A Study of Creatinine Elimination.—It has been established that a normal person ingesting a creatinine-free diet will excrete a uniform quantity of creatinine from day to day. The daily excretion of an adult man of average weight ranges from 1–1.5 grams. For data as to creatinine excretion of a 60 kg. man see Taylor and Rose's figures in table on page 857. The creatinine excretion depends primarily on the active mass of protoplasmic tissue, and therefore, it is generally true that a fat man will show a lower creatinine output than a lean man of like body weight. For further discussion of creatinine see Chapter XXVII.

Experiment.—Ingest an ordinary mixed diet (non-meat) for a period of three days varying the character of the diet daily. Collect the urine and analyze for creatinine. (See Chapter XXXI for methods of analysis.)

Did the creatinine elimination change with the change in diet?

9. Synthesis of Hippuric Acid in Human Body.—Hippuric Acid is present in human urine in small amount, about 0.7 gram being excreted per day. The urine of herbivorous animals contains much larger quantities. This acid is formed in the animal body, by synthesis from benzoic acid and glycocoll which takes place in the kidneys and elsewhere.[1]

Experiment.—Ingest 2 grams of sodium benzoate or ammonium benzoate before retiring at night. Collect the first fraction of urine voided the next morning. The benzoate has been synthesized with glycocoll to form hippuric acid. The urine will therefore be found to contain much more of this acid than is normally present. Isolate the hippuric acid by one of the following methods:

(*a*) *First Method.*—Render the urine alkaline with milk of lime, boil for a few moments and filter while hot. Concentrate the filtrate, over a burner, to a small volume. Cool the solution, acidify it strongly with concentrated hydrochloric acid and stand it in a cool place for 24 hours. Filter off the crystals of hippuric acid which have formed and wash them with a little cold water. Remove the crystals from the paper, dissolve them in a very small amount of hot water and percolate the hot solution through thoroughly washed animal charcoal, being careful to wash out the last portion of the hippuric acid solution with hot water. Filter, concentrate the filtrate to a small volume and stand it aside for crystallization. Examine the crystals under the microscope and compare them with those in Fig. 191, page 613. This method is not as satisfactory as Roaf's method (see below).

(*b*) *Roaf's Method.*—Place the urine in a casserole or precipitating jar and add an equal volume of a saturated solution of ammonium sulphate and 1.5 c.c. of concentrated sulphuric acid per 100 c.c. of urine. Permit the mixture to stand for

[1] Kingsbury and Bell: *Jour. Biol. Chem.*, 21, 297, 1915.

twenty-four hours and remove the crystals of hippuric acid by filtration. Purify the crystals by recrystallization according to the directions given above under First Method. Examine the crystals under the microscope and compare them with those given in Fig. 191, page 613.

It is possible, by the above technic, to isolate hippuric acid in crystalline form from as small a volume as 25–50 c.c. of herbivorous urine. The greater the amount of ammonium sulphate added the more rapid the crystallization until at the saturation point the crystals of hippuric acid sometimes form in about *ten minutes*.

10. The "Partition" of Urinary Nitrogen and Sulphur as Influenced by Diet.—It was first shown by Folin[1] that the percentage of the total nitrogen and total sulphur of the urine which appeared in the form of any particular nitrogenous constituent or in any particular form of sulphur was regulated directly by the extent of the total nitrogen and sulphur elimination. This point is well illustrated in the following table which contains data regarding the so-called "partition" or "distribution" of the urinary nitrogen and sulphur.

THE NITROGEN AND SULPHUR "PARTITIONS" AS INFLUENCED BY DIET[2]

Constituent of the urine	Normal protein diet			Starch-cream diet		
	Weight, grams	Nitrogen, grams	Per cent of total N or S	Weight, grams	Nitrogen, grams	Per cent of total N or S
Urea	31.6	14.7	87.5	4.72	2.2	61.7
Ammonia	0.6	0.49	3.0	0.51	0.42	11.3
Creatinine	1.55	0.58	3.6	1.61	0.60	17.2
Uric acid	0.54	0.18	1.1	0.27	0.09	2.5
Undetermined	.....	0.85	4.9	.....	0.27	7.5
Total N	.....	16.8	100.0	.....	3.6	100.0
Inorganic SO_3	3.27	.....	90.0	0.46	.....	60.5
Ethereal SO_3	0.19	.....	5.2	0.10	.....	13.2
Neutral SO_3	0.18	.....	4.8	0.20	.....	26.3
Total SO_3	3.64	.....	100.0	0.76	.....	100.0

[1] Folin: *Am. Jour. Physiol.*, 13, 118, 1905.
[2] Folin: *Am. Journ. Physiol.*, 13, 118, 1905.

It will be observed from an examination of this table that a normal protein diet which gave 16.8 grams of urinary nitrogen yielded 87.5 per cent of this nitrogen as urea, 3 per cent as ammonia, 3.6 per cent as creatinine and 1.1 per cent as uric acid; whereas a "non-protein diet" (starch and cream containing about 1 gram of nitrogen) which gave only 3.6 grams of urinary nitrogen yielded only 61.7 per cent of this nitrogen as urea but gave a greatly *increased percentage output* in the case of each of the other nitrogenous constituents mentioned, *e.g.*, 11.3 per cent as ammonia, 17.2 per cent as creatinine and 2.5 per cent as uric acid. The *percentage* output of neutral sulphur was also *greatly increased.*

It will furthermore be observed that the *actual daily output* of certain of the constituents is uninfluenced by the amount of protein ingested. Among these are creatinine and neutral sulphur. On the other hand the output of inorganic sulphur and urea is more or less directly proportional to the protein ingestion. The observation of such facts as these led Folin to formulate his theory of protein metabolism.[1]

Experiment.—During a period of two or three days ingest an ordinary mixed diet containing 100–125 grams of protein (16–20 grams of nitrogen) per day. Collect the urine accurately in 24-hour periods (page 789) preserve it and analyze the urine of the second and third days for total nitrogen, urea, creatinine, total sulphur, inorganic sulphates, ethereal sulphates and neutral sulphur (by difference). For methods of analysis see Chapter XXXI. Follow this period by one of three days in which a diet of starch and cream having a similar calorific value is ingested. Analyze the urine for the second and third days as indicated above. Calculate your results and tabulate as shown in the table on page 860. How did the change in the diet alter the metabolism of nitrogen and sulphur?

In calculating the calorific value of a diet make use of the following values:

1 gram protein.....4.1 large calories
1 gram fat.....9.3 large calories
1 gram carbohydrate.....4.1 large calories.

11. **Bacterial Nitrogen in Feces.**—About 50 per cent of the total nitrogen of the feces is made up of bacterial cells (see Chapter XVI on Feces). To demonstrate this point proceed as follows:

(*a*) Ingest an ordinary mixed diet. Collect a representative stool from this diet and after mixing it thoroughly separate the bacterial cells from a weighed portion as described in Chapter XVI. After examining some of the suspension under the microscope and noting the bacterial cells determine the bacterial nitrogen in the entire volume of suspension by the Kjeldahl method[2] (see Chapter XXXI). Also determine the total nitrogen in weighed portions of the original feces by the

[1] Folin: *American Journal Physiol.*, 13, 118, 1905.

[2] More accurate results will be secured if the bacterial nitrogen is determined on each individual stool in the fresh condition.

Kjeldahl method. What percentage of the total nitrogen of the feces is bacterial nitrogen?

(*b*) If it is desired to determine the actual *amount* of nitrogen which is excreted daily in the feces in the form of bacterial cells, proceed as follows: Ingest an ordinary mixed diet for a period of three days. Separate the feces for this period according to directions given on page 791, using charcoal for the first separation and carmine for the second or *vice versa*. Preserve the feces for the period according to directions given on page 790. Mix the weighed feces thoroughly and analyze for bacterial nitrogen and total nitrogen according to directions given elsewhere (see Chapters XVI and XXXI). Calculate the actual output of bacterial nitrogen per day and the percentage of the total nitrogen of the feces which was excreted per day in the form of bacterial nitrogen.

12. **"Metabolic Product" Nitrogen in Feces.**—A certain quota of the fecal nitrogen is due to the presence of residues of digestive secretions, epithelial cells, bacteria, etc. The nitrogen in these forms has been called "metabolic nitrogen." To determine this form of nitrogen one method[1] of procedure is as follows: Ingest a non-nitrogenous diet for a period of two days. The diet may include desired quantities of *starch, cream, sugar, butter, water and sodium chloride.* About 15 grams of *agar-agar* should be added to the diet to prevent constipation and to insure the evacuation of approximately the normal quantity of feces. (For influence of agar-agar see Exp. 5, p. 792.) To separate the feces properly ingest a capsule of carmine at the beginning of the test and one of charcoal at the end (see page 791). Preserve the feces as described on page 790. After mixing the feces thoroughly determine the nitrogen in weighed quantities by the Kjeldahl method[2] according to directions given in Chapter XXVII. Calculate the quantity of nitrogen eliminated per day. Inasmuch as no nitrogen was ingested the nitrogen present in the feces is of metabolic origin, *i.e.*, it is made up principally of nitrogen in the form of cells, digestive secretions and bacteria.

13. **Influence of Defective Mastication on Food Residues in Feces.**—Rapid eating accompanied by defective mastication leads to the appearance of relatively large macroscopic food residues in the feces. Under some conditions, however, *protein utilization* (see above) may be as satisfactory during food "bolting" as when the food is very thoroughly masticated.[3] This problem may be studied by the following method:

(*a*) Ingest a diet containing meat and be certain to masticate the diet very thoroughly. Collect a stool, examine macroscopically; mix carefully and examine microscopically (see pages 317—318).

(*b*) Ingest a diet similar to that employed in above experiment (*a*). "Bolt" the food, *i.e.*, ingest it practically without mastication. Examine the feces as above. Note the difference in the macroscopical and microscopical findings under (*a*) and (*b*).

If the nitrogen of food and feces is determined we may calculate the protein utilization (see Exp. 2, p. 851). By the additional determination of urinary nitrogen, we may prepare a nitrogen balance (see Exp. 6, page 870).

[1] For a discussion of other methods of estimating metabolic product nitrogen see Forbes Mangels, and Morgan: *Jour. Agr. Res.*, 9, 405, 1917.

[2] In the oxidation process use 10 grams of potassium sulphate instead of the copper sulphate. The remainder of the procedure is the same as for urine.

[3] Foster and Hawk: *Jour. Amer. Chem. Soc.*, 37, 1347, 1915.

IV. EXPERIMENTS ON CARBOHYDRATE METABOLISM

1. Hyperglycemia Produced by Carbohydrate Ingestion.—The average glucose content of normal blood is somewhat less than 0.1 per cent. This is increased (hyperglycemia) on the ingestion of carbohydrate food. The increase is noted more quickly after the ingestion of monosaccharides than after the ingestion of the more complex carbohydrates. After the ingestion of 100 grams of glucose or starch an increase in the sugar of the blood sometimes occurs in five minutes.[1] (See Fig. 270.)

(a) Influence of Glucose.—In the morning before breakfast, or three to five hours after breakfast, determine the normal sugar content of your blood by means of some accurate micromethod. (See Chapter XVIII.) Ingest 100 grams of glucose dissolved in 250 c.c. of water, and again determine the blood sugar level at intervals of 5, 15 and 30 minutes and one, two and three hours. (Plot a curve similar to the one shown in Fig. 270.) The urine may also be examined for sugar at intervals of one hour after the sugar ingestion.

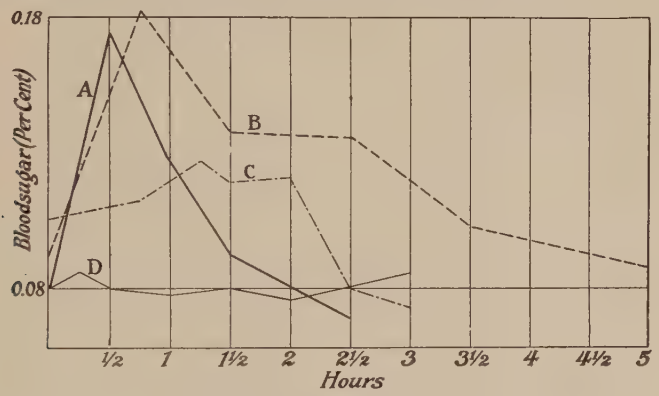


FIG. 270.—BLOOD SUGAR AS INFLUENCED BY DIET.
A = glucose; *B* = starch;
C = starch and fat; *D* = fat.

Repeat the experiment on another day using 250 grams of glucose and compare the results with those obtained after the ingestion of 100 grams. Explain your findings. If desired this experiment may be combined with the ones on "Alimentary Glycosuria," and "Carbohydrate in Feces," see pages 865 and 866.

(b) Influence of Starch.—Repeat the experiment as given above for glucose except that 170 grams of white bread or 100 grams of starch made into a paste[2] are substituted for the 100 grams of glucose.

The experiment may be repeated as described above using an increased amount of starch.

The various experiments may be conducted on patients suffering from diabetis mellitus if such are available and instructive data

[1] Jacobson: *Bioch. Zeit.*, 56, 471, 1913.

[2] In making starch paste, rub up the dry starch in a mortar with cold water and pour the suspended starch granules into boiling water and stir.

collected. The alimentary hyperglycemia will generally be slower in reaching its maximum and will be more prolonged than in the case of normal subjects. In some instances (see Fig. 120, p. 389) after the diabetic has ingested 100 grams of glucose the blood sugar does not reach its maximum until a period of two hours has elapsed.[1] The blood sugar also returns to its former level more slowly than in the case of normal individuals.

2. The Influence of Fasting on Carbohydrate Utilization.[2]—Two or more healthy rabbits are fasted for periods of one, 4 or more days. Carbohydrate tolerance tests are then performed.

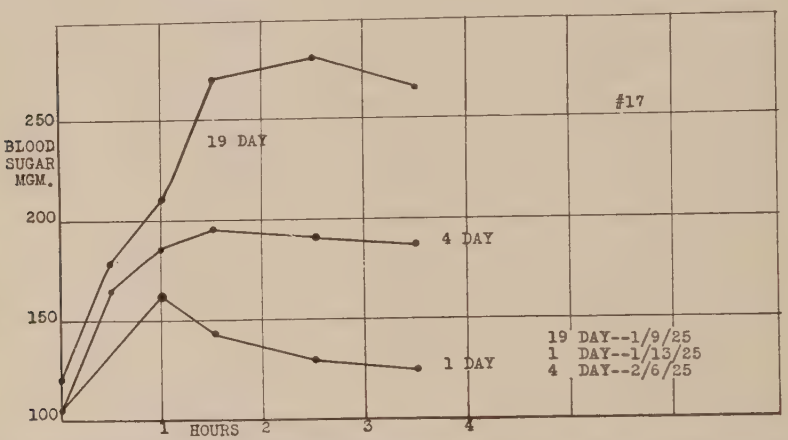


FIG. 271.—INFLUENCE OF FASTING ON CARBOHYDRATE UTILIZATION. (*du Vigneaud and Karr: Jour. Biol. Chem.*, 66, 281, 1925.)

Shave the edge of one ear, wash with alcohol and dry. Cut the marginal vein with a scalpel. Draw 30 drops of blood[3] into an oxalated tube. Introduce through a stomach tube[4] 3 gm. glucose per kg. body weight, dissolved in about 35 c.c. of water. Blood is then drawn (from the same incision if possible) at half hourly intervals, for one and a half hours, and then at hourly intervals for two hours. The samples of blood are then analyzed for glucose by one of the methods described on p. 381 *et seq.* Compare results with those shown in Fig. 271, and observe that *fasting decreases the tolerance for carbohydrate.*

[1] Martin and Mason: *American Journal of Medical Sciences*, 153, 50, 1917.

[2] du Vigneaud and Karr: *Jour. Biol. Chem.*, 66, 281, 1925.

[3] The circulation in the ear may be stimulated by mechanical means (rubbing), or by touching the tip with a drop of xylol. After the blood is collected, the xylol must be washed off with alcohol to prevent an eczematous condition.

[4] A soft rubber catheter about 15 inches long, to which is connected a small funnel, may be used for this purpose. The rabbit's jaws are held open by means of a wooden block through which passes a hole to admit the tube. It is necessary to hold the animal's head and body firmly while passing the tube. Before introducing the glucose solution, ascertain whether the tube is in the stomach and not in the lung by holding the end close to the ear and listening for respiratory sounds.

3. The Influence of Diet on Carbohydrate Utilization.[1]—Four healthy rabbits are fasted for a period of 4 days. Keep one as normal control, and to the others introduce by stomach tube (see p. 864) respectively, 10 gm. of lard oil, glucose and egg white. On the following day (18 hours later) conduct glucose tolerance tests on all four rabbits, as described in the above experiment. Compare results with those shown in Fig. 272. Observe the variations in the effects of car-

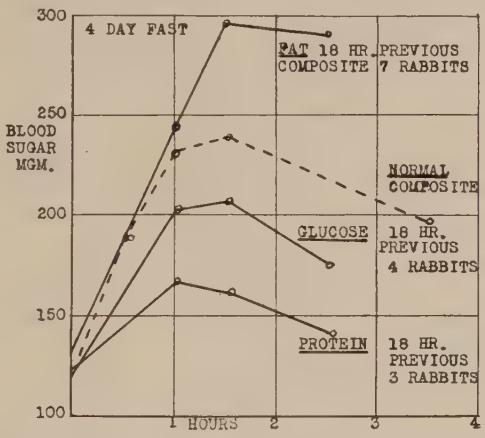


FIG. 272.—INFLUENCE OF DIET ON CARBOHYDRATE UTILIZATION. (*du Vigneaud and Karr: Jour. Biol. Chem.*, 66, 281, 1925.)

bohydrate, protein and fat in the previous diet, on the capacity to utilize glucose.

4. Experiments on Alimentary Glycosuria.—According to Folin and Berglund[2] the sugar of normal urine consists of a motley variety of carbohydrate products and carbohydrate derivatives including di- and polysaccharides. These are believed to arise from foreign unusable carbohydrate materials present in grains, vegetables, and fruits, and from decomposition products due to cooking, canning, and baking of food. The ingestion of pure glucose, fructose, maltose, dextrin, or starch does not normally give rise to glycuresis, but the ingestion of impure products or of an ordinary carbohydrate meal increases the sugar of the urine. So also do lactose and galactose.

Experiment.—On arising at 7:00 A. M., the student should empty his bladder and discard the urine voided. He should then drink one glass of water but eat no food. At 8:00 A. M. the bladder is again emptied and the urine kept for analysis. The student then immediately drinks 200 grams of pure glucose dissolved in about 500 c.c. of water. Urine specimens are collected again at 9:00, 10:00 and 11:00 A. M. Test each specimen for sugar by Benedict's or other qualitative reduction test. Determine free and total sugar in each specimen according to the method of Folin and Berglund or total and non-fermentable

[1] duVigneaud and Karr: *Jour. Biol. Chem.*, 66, 281, 1925.

[2] Folin and Berglund: *Jour. Biol Chem.*, 51, 213, 1922.

sugar according to the methods of Benedict and Osterberg (see Chapter XXXI for methods).

Other students should go through the same procedure with the exception that for glucose there should be substituted 200 grams of cane sugar, 200 grams commercial dextrin, 200 grams pure dextrin-starch in a mixture produced by boiling 200 grams starch with 900 c.c. of water, cooling to 50°C., adding a little extract of malt, and allowing to digest until liquid, 100 grams pure lactose, a dozen graham crackers with water only, two baked apples, bread and butter, meat with water only or a large dish of pure gelatin without added sugar.

This experiment may be made more complete by making determinations of blood sugar at short intervals as described in Experiment 1, page 863. If desired, data on glycosuria, hyperglycemia and carbohydrate in feces (below) may be collected from one experiment.

5. Absorption of Carbohydrate as Influenced by Fat Ingestion.—When fat is eaten along with carbohydrate food the absorption of the latter is somewhat delayed. This has been shown experimentally.[1] To demonstrate the point proceed as follows: Determine the content of sugar in the blood at various intervals after the ingestion of 170 grams of white bread as described in Experiment 1 (b), page 863. Plot a curve for these values similar to the one shown in Fig. 271, page 864. On a later day repeat the experiment and ingest 170 grams of white bread and 85 grams of butter. Plot the curve for these blood sugar concentrations along with blood sugar values obtained after the ingestion of white bread as described above. Has the fat exerted any influence upon the absorption of the carbohydrate? Repeat the above experiment on a case of diabetes mellitus if such is available and note that fat exerts the same influence upon carbohydrate absorption as it exerts in the normal human body.

6. Carbohydrate in Feces.—Under normal conditions the great bulk of the soluble carbohydrate in the food is absorbed from the intestine even when the ingestion is high. Hence the content of soluble carbohydrate in feces is low. To demonstrate this proceed as follows:

(a) Ingest for three days an ordinary mixed diet to which 100 grams of glucose or sucrose is daily added. Separate and preserve the feces (see page 790) and when the final "marker" appears extract an aliquot portion of the total mixed feces[2] with water, decolorize with boneblack, filter, and after making the filtrate up to a known volume determine the sugar by Benedict's method (see page 641). Calculate the soluble carbohydrate content of the feces for the three-day interval. (b) Proceed as above with the exception that at least 250 grams of sugar should be added to the diet instead of 100 as in (a).

How did the daily excretion of soluble carbohydrate in (a) compare with that in (b)? Why is this so? If a diet of known carbohydrate content is fed this experiment will give us accurate data as to soluble carbohydrate utilization (see Protein Utilization, page 851). If it is desired this experiment may be combined with the hyperglycemia and glycosuria experiments on pages 863 and 865. See also Experiment 7, page 867.

[1] Jacobson: *Bioch. Zeit.*, 56, 471, 1913.

[2] If time permits it is more satisfactory to analyze each individual stool in the fresh condition.

7. A Study of the Elimination of Carbohydrate in Urine and Feces after Excessive Carbohydrate Ingestion.—Combine the procedures outlined in Experiments 4 and 6, pages 865 and 866.

8. Effect of Exercise on the Composition of the Urine.[1]—The subject who has had no food for several hours is given 50 c.c. of water every 15 minutes throughout the experiment beginning for example at 12:00 noon. At 12:00 the bladder is emptied and urine specimens collected every 15 minutes thereafter. At 1:00 the subject engages in strenuous exercise for 2–3 minutes. Running up and down stairs for this period

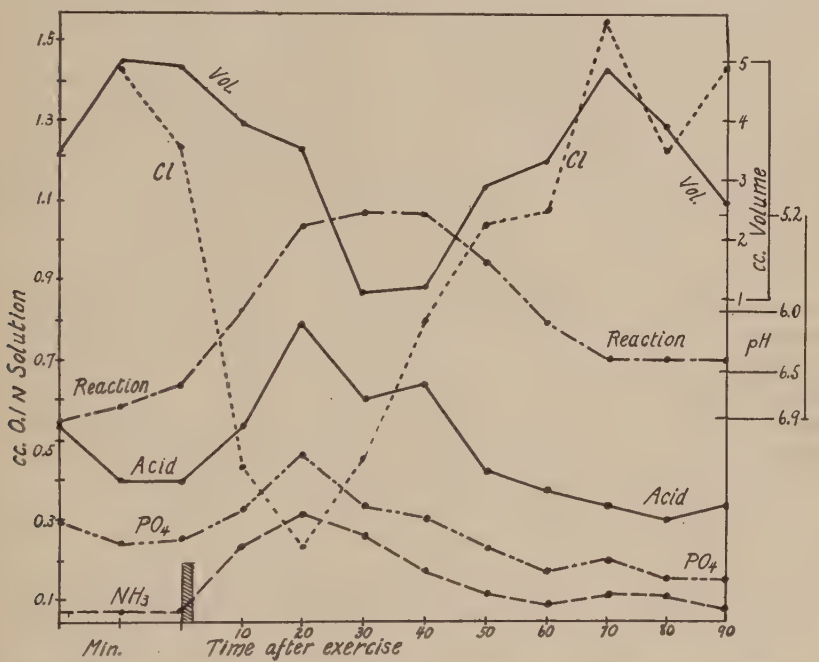


FIG. 273.—CURVES SHOWING INFLUENCE OF EXERCISE ON COMPOSITION OF THE URINE. (*Wilson, et al: Jour. Biol. Chem.*, 65, 755, 1925.)

will suffice. In the urine specimens determine lactic acid (p. 743) phosphate (p. 776) total acidity (p. 705) chlorine (p. 780) ammonia (p. 724) and pH (p. 707). Plot curves of amounts of these per 10 minute specimen against time to show the effect of exercise on urine composition. Urine volumes generally decrease, acidity and ammonia increase. The phosphate and lactic acid excretions increase while the chloride excretion decreases. The maximum effect is usually obtained in 20 minutes. Normal values should be restored in about an hour. The blood sugar may also be determined at intervals and the influence of the exercise noted. Curves from a typical experiment are shown in Fig. 273.

[1] Liljestrand and Wilson: *Jour. Biol. Chem.*, 65, 773, 1925. Wilson, Long, Thompson, and Thurlow: *Jour. Biol. Chem.*, 65, 755, 1925.

V. EXPERIMENTS ON FAT METABOLISM

1. **Fat Utilization.**—This may be determined in a manner entirely analogous to that used in the determination of protein utilization (see p. 851). The fat may be determined by the Saxon Method (Chapter XVI). It is claimed when moderate amounts of fat are fed that the fat of the feces is largely independent of the diet. Therefore, in order to secure accurate information regarding the utilization of food fat, it is said to be necessary to determine the fecal fat on a non-fat diet. There may possibly be a fat excretion from the intestine.[1] (See Chap. XVI.)

2. **Fat in Feces.**—A normal adult will digest and absorb at least 90 per cent of the fat in the diet when the amount ingested does not exceed 100 grams. If the diet contains an excessive amount of fat, *e.g.*, 300 grams per day, considerable appears in the feces. In pancreatic diseases and such conditions as are accompanied by a decrease in bile flow the digestion and assimilation of fat is lessened.

Experiments.—(a) Ingest an ordinary mixed diet containing an average amount of fat per day, e.g., 75–100 grams. Collect a stool and examine it microscopically as directed in Chapter XVI. (b) Now ingest a diet containing an excessive quantity of fat, e.g., 300 grams per day. Separate the feces and subject a representative sample of the feces from the high fat diet to microscopical examination. (c) If it is desired the fat may be extracted from some of the stool by applying the principle involved in the quantitative determination of fat in the Saxon method (see Chapter XVI). Evaporate the ether extract and identify the fat in the residue by tests given in Chapter X.

VI. EXPERIMENTS ON INORGANIC METABOLISM

1. **"Salt-free" Diet.**—In order to be properly nourished we must ingest a certain amount of inorganic matter daily. If we fail to do this our metabolic processes become abnormal and the urine is one index of this abnormality.[2]

Experiment.—Ingest an ordinary mixed diet containing an ample salt content for a period of two days. Follow this period by the ingestion of a diet which has had its salt content reduced to a very low value.[3] Sugar and olive oil or non-salted butter may supply the bulk of the calorific part of the diet and dialyzed egg white or casein or commercial protein preparations, e.g., plasmon, gluten or glidine may supply the protein. Ingest such a diet for three days. (This is an "acid-forming" diet, see page 875.) Collect the urine and analyze for sodium chloride, acidity, ammonia and total nitrogen. Compare the data from the normal days

[1] Hill and Bloor: *Jour. Biol. Chem.*, 53, 1, 1922.
[2] Taylor: University of California Publications, Pathology, 1.
[3] It is practically impossible to secure an absolutely "salt-free" diet

with those obtained when the "salt-free" diet was ingested. Test the urine (Chapter XXXI) and blood (Chapter XVIII) for acetone. An acidosis follows the ingestion of a salt-free diet for a sufficient length of time.

Did you feel perfectly normal during the interval you were ingesting the "salt-free" diet?

2. Salt-rich Diet.—On an ordinary mixed diet a normal adult will daily excrete 10–15 grams of chloride, expressed as sodium chloride, in the urine. On a salt-free diet this excretion decreases, whereas if the diet contains an excessive quantity of sodium chloride this excess will be promptly excreted in the urine. Normal feces contain very little sodium chloride even after excessive sodium chloride ingestion (see Exp. 4, below).

Experiment.—Ingest an ordinary mixed diet for two days. On each of the following two days take a similar diet plus a weighed amount (e.g., 10 grams) of sodium chloride. Collect the urine for the four days in 24-hour samples, preserve and analyze for sodium chloride (for methods see Chapter XXXI). What proportion of the added chloride was recovered?

If it is desired to make the experiment quantitative in character ingest a uniform diet (see Experiment, page 851) each day instead of the ordinary mixed diet, and examine urine and feces (see Exp. 4 below) for chloride.

3. Ultraviolet Irradiation.—For experiments demonstrating the influence of ultraviolet light on inorganic (calcium) metabolism, see p. 829.

4. Inorganic Elements in the Feces.—The salts of sodium and potassium being very soluble are almost completely absorbed from the intestine. The same is true of the chlorides including that of sodium which is of greatest importance. Hence the alkali metals and chlorides are excreted mainly in the urine and are found only in very small amounts in the feces even when large amounts are ingested. With calcium, magnesium, iron and phosphate conditions are different. Not only are salts of calcium, magnesium and iron less readily absorbed but they are excreted to a large extent by way of the intestinal mucosa rather than by the kidneys. Ordinarily about 90 per cent of ingested calcium is eliminated by way of the feces and a little less than half of the magnesium. From 20–30 per cent of the phosphorus ingested is usually found in the feces.

Experiments.—(a) Ingest for a period of three days an ordinary mixed diet without added salt and containing no milk. Separate the feces for the period (see page 791) and retain a portion of the well-mixed feces for analysis.

(b) Proceed as above with the exception that there is added to the mixed diet 10 grams of common salt and a quart of milk (containing about 1.6 grams of CaO, 0.2 gram MgO, 1.4 grams of chloride expressed as sodium chloride, and 2.2 grams P_2O_5). Mix feces well and reserve part for analysis.

Ash 10 gram samples of the feces from the above diets. Dissolve with the aid of a little dilute nitric acid, filter and make up to 100 c.c. Determine in aliquot portions of this solution: (1) Chlorides by Volhard method. (2) Calcium and magnesium by McCrudden's method. (3) Phosphorus by uranium titration. (For details of analytical methods see Chapter XXXI.) Calculate the percentages of the added Ca, Mg, P, and Cl which are recovered from the feces.

For a more detailed study of chloride excretion combine this experiment and Exp. 2, p. 869 (see Exp. 6, below).

5. Excretion of Urinary and Fecal Chloride after a High Chloride Ingestion.—Combine the procedures outlined under Experiments 2 and 4, page 869.

6. Preparation of a Metabolic Balance.—This test entails the analysis of the food ingested and of the urine and feces excreted, *i.e.*, a study of the *income* and *outgo*. Proceed as follows:

Select a diet which is simple, i.e., consists of few constituents, and which lends itself readily to accurate chemical analysis. A good type of diet for ordinary metabolism experiments of this sort consists of crackers (graham or soda), milk, butter, water and agar-agar (to prevent constipation). Meat specially prepared in quantity sufficient for an entire experiment may also be utilized. Ingest uniform quantities of these dietary constituents each day for a period of three days.[1] Make an accurate collection of the urine passed during this interval (see page 789). Separate the feces representing the three-day period (see page 791), and analyze foods, urine and feces. The balances ordinarily prepared are those for nitrogen, sulphur, phosphorus and calcium. Analytical methods for the determination of these elements may be found in Chapter XXXI.

BALANCE OF CALCIUM, MAGNESIUM, PHOSPHORUS, SULPHUR, AND NITROGEN IN ACROMEGALY

	Calcium oxide	Magnesium oxide	Phosphoric anhydride	Sulphur	Nitrogen
	Grams				
Ingestion (daily)	1.494	0.486	3.192	1.190	18.84
Excretion (urine)	0.159	0.160	1.701	1.006	17.60
Excretion (feces)	1.093	0.226	1.002	0.135	1.10
Excretion (total)	1.252	0.386	2.703	1.141	18.70
Retention (daily)	0.242	0.100	0.489	0.049	0.14
Retention (per cent)	16.2	20.6	15.3	4.1	0.7

The foregoing table includes balances obtained in a metabolism test on acromegaly.[2]

[1] See note 1, p. 792.

[2] Bergeim, Stewart and Hawk: *Jour. Expt., Med.*, 20, 218, 1914.

VII. EXPERIMENTS ON THE INFLUENCE OF WATER ON METABOLISM

It has been demonstrated that increased water ingestion influences favorably many of the functions and activities of the human body.[1] The increase in protein catabolism which accompanies high-water intake is shown in the following data collected from an experiment upon a normal man.[2] In this experiment the water ingestion *at meals* was increased 3 liters per day during the Water Period.

INFLUENCE OF HIGH-WATER INTAKE UPON URINE VOLUME AND NITROGEN PARTITION

Day of experiment	Urine volume	Nitrogen	Urea-nitrogen	Ammonia-nitrogen	Creatinine-nitrogen	Creatine-nitrogen
			Preliminary Period			
	c.c.	grams	grams	grams	grams	grams
4	830	12.987	11.338	0.288	0.629	.....
5	920	12.084	11.476	0.305	0.619	.....
6	880	13.183	11.568	0.369	0.651	.....
			Water Period			
7	3440	14.161	12.596	0.486	0.610	0.063
8	3840	13.491	11.583	0.499	0.616	0.024
9	3670	12.981	11.212	0.553	0.589	0.102
10	3610	12.976	11.455	0.485	0.608	0.055
11	4020	13.138	11.879	0.456	0.589	0.128

The above data indicate an increased catabolism of protein material as is shown by an increased output of total nitrogen upon the first and second days (days 7 and 8) of the Water Period. Part of this increase may, however, have been due to a "flushing" of the tissues rather than to increased catabolism of protein structures.

(1) **Relation of Water Intake to Volume and Specific Gravity of the Urine.**—Ingest an ordinary mixed diet for two days. Collect the urine in 24-hour periods. During the first day ingest very little fluid of any kind either at meals or between

[1] Hawk: The relationship of water to certain life processes and more especially to nutrition. Read before *American Philosophical Society*, Philadelphia, Feb., 1914. (See *Bioch. Bull.*, 3, 420, 1914.) The so-called "water intoxication" demonstrated by Rowntree (*Arch. Int. Med*, 32, 157, 1923) and later by Underhill and Sallick (*Jour. Biol. Chem.*, 63, 61, 1925) cannot be advanced as an argument against the value of a high water intake for the average individual. The observers mentioned experimented on animals and introduced as much as 50 c.c. of water per kg. body wt. every half hour until a definite toxic result was obtained. A similar excessive ingestion of any standard food would probably produce fully as serious results.

[2] **Fowler and Hawk:** *Jour. Expt Med.* 12, 388, 1910.

meals. On the second day ingest as much water as you can without physical inconvenience. A person of average size should have no difficulty in drinking 5–6 quarts per day.

Measure the volume of each day's urine and take the specific gravity. Note the pronounced increase in volume and the low specific gravity of the urine under the influence of high-water ingestion.

(2) Influence on Protein Catabolism.—That water stimulates protein catabolism may easily be demonstrated as follows: Ingest a uniform diet (milk, crackers, butter, peanut butter and water) for a period of four days. During the first two days ingest your customary volume of water per day. During the last two days increase the water ingestion to 5–6 liters per day. Collect urine in 24-hour periods and analyze for total nitrogen by Kjeldahl method (see Chapter XXXI and note on page 546). Note the increased excretion of nitrogen under the influence of high-water intake. If time permits other nitrogenous urinary constituents may be determined (see table above).

VIII. EXPERIMENTS ON ACID-BASE METABOLISM

1. **Ketosis.**—Ketosis may be induced in a normal person by the ingestion of a "salt-free" diet such as described in Exp. 1, p. 868, above, or by the ingestion of a carbohydrate-free diet. The ketosis appears somewhat earlier under the latter conditions. The non-carbohydrate diet is rather better suited for the demonstration because of its greater palatability. When carbohydrates are ingested there is an oxidation of fatty acids to carbon dioxide and water. When no carbohydrates are ingested a portion of the fatty acids are converted into *acetone bodies*. These are difficult to oxidize and are excreted as such. The *ketonuria* (excretion of acetone and diacetic acid) is particularly pronounced. The following table shows the data obtained in an actual case of the withdrawal of carbohydrate food from the diet of a normal man (von Noorden).

ACIDOSIS ACCOMPANYING CARBOHYDRATE WITHDRAWAL

Day	Diet	Excretion of acetone bodies calculated as β-hydroxybutyric acid (grams)
1	Protein, fat and *carbohydrate.*	None.
2	Protein and fat.	0.8
3	Protein and fat.	1.9
4	Protein and fat.	8.7
5	Protein and fat.	20.0
6	Protein, fat and *carbohydrate.*	2.2

Experiment.—Ingest an ordinary mixed diet for one day. Follow this by a period of two to four days in which no digestible carbohydrate is eaten. (A diet of meat, eggs, butter, agar-agar and water has a very low digestible carbohydrate value.) Collect the urine for each day of the experiment, examine it qualitatively for acetone bodies (see tests in Chapter XXVIII). If present, determine the total acetone bodies quantitatively (for methods see Chapter XXXI). The blood may also be examined (see Chapter XVIII). Did the withdrawal of carbohydrate food cause an acidosis or ketonuria? How did it compare with the acidosis in the above table?

The role of various dietary constituents in enhancing the production of acetone bodies (*ketogenesis*) or inhibiting it (*antiketogenesis*) has given rise to the classification of dietary constituents on this basis.[1] Thus we have the *ketogenic* substances, fatty acids and certain amino acids like leucine, tyrosine, phenylalanine and possibly histidine, and the *antiketogenic* substances, glycerol, glucose and sugar-forming amino acids, like glycocoll, and alanine.

Attempts have been made to explain the antiketogenic nature of carbohydrates on the basis of a chemical reaction between some fragment of the glucose molecule and one of the acetone bodies, acetoacetic acid. As a result, more or less definite ratios must exist between the ketogenic and the antiketogenic factors in the diet, if ketosis is to be averted. Shaffer[2] who has contributed much to this subject, has evaluated carbohydrates, proteins and fats according to their equivalence in terms of acetoacetic acid and glucose. In the case of man, he found a close agreement with experimental facts when it was assumed that two molecules of acetoacetic acid reacted with one of glucose. If the ratio of ketogenic to antiketogenic molecules is greater than 2:1, acetone bodies are excreted: if it is less than 2:1, acetonuria is absent or is very slight. The food mixture undergoing metabolism was determined from the urinary nitrogen excretion together with the results of direct and indirect calorimetry. These deductions have an important bearing on the feeding of diabetics.

Other investigators, working chiefly with lower animals, have found cause to disagree with Shaffer's principle of a fixed ratio between ketogenic and antiketogenic factors in the diet, and the occurrence of a molecular reaction between acetoacetic acid and glucose, which Shaffer demonstrated *in vitro*. Leathes[3] believes that some of the assumptions which have been made, and admitted by Shaffer, are not founded on sufficient evidence. However the applicability of the ketogenic-anti-

[1] Geelmuyden: *Ergeb. d. Physiol.*, 22, 51, 1923.

[2] Schaffer: *Jour Biol. Chem.*, 47, 433 and 449, 1921; 49, 143, 1921; 54, 399, 1922; 61, 585, 1924 Also Harvey Society Lecture, 1923–24

[3] Leathes and Raper, The Fats, Longmans, Green and Co., London and New York, 2nd ed., 1925.

ketogenic (K/A) ratio to humans has been demonstrated, and proven useful. It is possible that lower animals have higher urinary thresholds for acetone bodies, or that the biological factor of the living organism influences the molecular relationships.[1]

Shaffer's calculation of the K/A ratio, and the expected excretion of acetone bodies ("Ketogenic balance") follows:

Ketogenic Equivalent (as millimols acetoacetic acid)

Protein	$= a$ gm. Urinary N $\times$ 15
Fat	$= b$ gm. Fat $\times$ 3.43

Antiketogenic Equivalent (as millimols glucose)

Carbohydrate	$= c$ gm. Glucose $\times$ 5.56
Protein	$= d$ gm. Urinary N $\times$ 20
Fat	$= e$ gm. Fat $\times$ 0.57

$$\text{"Ketogenic-antiketogenic ratio"} = \frac{\text{Total ketogenic molecules}}{\text{Total antiketogenic molecules}} = \frac{a+b}{c+d+e}$$

$$\text{"Ketogenic balance"} = \text{Total ketogenic molecules} - (2 \times \text{total antiketogenic molecules}). = (a+b) - 2(c+d+e)$$

Excess ketogenic molecules $\times$ 0.104 = expected excretion of acetone bodies, as gm. β-hydroxybutyric acid.

Employing these factors, together with a knowledge of the total metabolism, the amount of carbohydrate ingested, the amount of fat burned and the nitrogen excreted, it is possible to calculate the K/A ratio and to determine the expected output of acetone bodies, as β-hydroxybutyric acid.

A more simple and convenient formula, especially when the urinary nitrogen excretion is not known, has been suggested by Ladd and Palmer:[2]

$$\frac{K}{A} = \frac{\text{gm. Fat}}{(0.58 \times \text{gm. Protein}) + \text{gm. Carbohydrate}}$$

When this ratio exceeds 4:1, ketosis is said to occur. The composition of the diet alone enters into this formula, but no account is made for the antiketogenic glycerol from fat, or of the ketogenic derivatives of the protein. It has been demonstrated[3] that normal growth may take place in animals which are fed highly ketogenic (high fat) diets.

[1] Allen: *Jour. Metab. Res.*, 3, 797, 1923; 4, 189, 199 and 223, 1923.
[2] Ladd and Palmer: *Am. Jour. Med. Sci.*, 166, 157, 1923.
[3] Osborne and Mendel: *Jour. Biol. Chem.*, 59, 13, 1924
Smith and Levine: *Jour Biol. Chem.*, 67, vi, 1926

Ketosis in the human, does not follow immediately upon changing to a high fat diet or in starvation, owing to the combustion of the glycogen stores in the body. When these are depleted, acetone bodies appear in the urine. The lower animals, *e.g.* the rat, appear to resist the onset of ketosis more than man.[1] The administration of sodium bicarbonate in cases of diabetic acidosis (ketosis) results in an increased excretion of acetone bodies ("washing out"), but appears to stimulate their formation by disturbing the fat metabolism. Hence glucose is usually given intravenously to provide antiketogenic substance, and thus restore the K/A ratio.

Injection of the drug phloridzin has been used in experimental animals, particularly the dog, to simulate the condition of diabetes. By this means ketosis may be produced along with the glycosuria.

2. "Alkaline Tide."—For a time after a meal the normal acid reaction of the urine may be changed to neutral or alkaline. This has been explained as due to the withdrawal of hydrogen ions to manufacture the hydrochloric acid of the gastric juice. The presence of an alkaline tide has been used as an indication of the secretion of hydrochloric acid in the stomach in cases where it was desirable to avoid passing the stomach tube. The urinary ammonia excretion may also serve a similar purpose.

Experiment.—Ingest an ordinary mixed diet. Urinate just before dinner and note the reaction of the urine to litmus. If acid, determine the hydrogen ion concentration by the method given in Chapter XXXI. (If alkaline, discard the urine and make the test on another day.) After eating a heavy dinner (meats) collect the urine at intervals of a half-hour and take the reaction to litmus and determine the hydrogen ion concentration as before. Did your urine change in reaction after the meal and if so how long a period elapsed between the meal and the occurrence of the maximum change in reaction?

If desired, a supplementary experiment may be conducted in which a test-meal is given and samples of gastric contents withdrawn at intervals by the Rehfuss tube and the acidity of these samples compared with the reaction of samples of urine voided at similar intervals.[2]

3. Hydrogen Ion Concentration of the Urine as Influenced by the Ingestion of Acid-Forming and Base-Forming Foods.—It has been demonstrated by Sherman and Gettler[3] that vegetables and fruits, on burning, leave an ash in which the basic elements (sodium, potassium, calcium and magnesium) predominate, whereas cereals, meats and fish foods leave an ash in which the acid-forming elements (chlorine, sulphur

[1] Smith and Levine: *Loc. cit.*
[2] Hubbard and Mumford: *Proc. Soc. Expt. Biol and Med.*, 19, 429, 1922.
[3] Sherman and Gettler: *Jour. Biol. Chem.*, 11, 323, 1912.

and phosphorus) predominate. A list of acid-forming and base-forming foods, is given in the following table. These values are in "general agreement" with the more recent work by Clark.[1]

EXCESS OF ACID-FORMING OR BASE-FORMING ELEMENTS IN FOODS
(Sherman and Gettler)

Article of Food	Excess acid or base in terms of normal solutions. Per 100 grams	
	Acid (c.c.)	Base (c.c.)
Apples		3.76
Asparagus		0.81
Bananas		5.56
Beans (dried)		23.87
Beans (lima, dried)		41.65
Beets		10.86
Cabbage		4.34
Cantaloup		7.47
Carrots		10.82
Cauliflower		5.33
Celery		7.78
Crackers	7.81	
Eggs	11.10	
Egg-white	5.24	
Egg-yolk	26.69	
Fish (haddock)	16.07	
Lemons		5.45
Lettuce		7.37
Meat (lean beef)	13.91	
Milk (cow's)		2.37
Oatmeal	12.93	
Oranges		5.61
Potatoes		7.19
Prunes		24.40[2]
Raisins		23.68
Rice	8.10	
Wheat (entire)	9.66	

The above data indicate that potatoes, oranges, raisins, apples, bananas and cantaloups are important base-forming foods. Among the most important acid-forming foods are found rice, whole wheat bread, oatmeal, meats and eggs. Certain fruits, *e.g.*, cranberries,[3] prunes and plums yield a basic ash but are acid-forming foods.

[1] Clark: *Jour. Biol. Chem.*, 65, 597, 1925.

[2] Prunes, plums and cranberries yield an alkaline ash but serve to *increase* the hydrogen ion concentration of the urine because of their benzoic acid content, this acid being synthesized with glycocoll in the kidney and elsewhere to form hippuric acid.

[3] Radin reports this berry to contain 0.06 per cent benzoic acid (Blatherwick: *Arch. Int. Med.*, 14, 409, 1914).

This is due to the fact that they contain benzoic acid which is synthesized with glycocoll in the body to produce hippuric acid (see page 859). It is worthy of note that some plant foods are base-formers and others are acid-formers. It is also an important fact that *acid* fruits yield a *basic* ash (see page 876).

The normal diet should contain sufficient base-forming elements to neutralize the acids formed. If these acids are not neutralized by the basic elements in the diet they will be neutralized by the fixed bases of the tissues of the body and a seriously deranged metabolism may result. (See experiment on "salt-free diet," page 868.) Organic salts of the alkalis (*e.g.*, sodium bicarbonate or sodium acetate) are often given therapeutically. They reduce the H ion concentration of the urine: the same result so far as urine reaction is concerned may be secured by feeding properly selected base-forming foods. The ingestion of sodium dihydrogen phosphate [NaH_2PO_4] will increase the acidity of the urine: a like result may be produced by feeding properly selected acid-forming foods. Anything which produces an increase in the H ion concentration of the urine will produce an increase in the ammonia output.

On a mixed diet the H ion concentration of the urine[1] has been found to average about pH 6.0.[2] In nephritis the pH of the urine may be decreased to 5.3 or less (see page 707). Alkalis have been used with apparent success in the treatment of nephritis.[3] It is evident that base-forming foods properly selected should be suitable dietary articles for nephritics.[3] For a detailed discussion of acid-forming and base-forming foods see article by Blatherwick.[4]

Experiment.—Ingest a uniform diet consisting of milk, crackers, butter, peanut butter, and water in desired quantities for a period of three days. Follow this by a period of six days during the first three of which considerable quantities of acid-forming foods (see table page 876) are added to the diet. During the second half of the period (days four to six) add an abundance of base-forming foods to the diet. Distilled water should be used for drinking purposes and a uniform volume should be ingested daily. Collect the urine in 24-hour periods, preserve and analyze for H ion concentration, titratable acidity and ammonia (for methods see Chapter XXXI). Compare your results with those tabulated in the table below.

[1] Henderson and Palmer: *Jour. Biol. Chem.*, 13, 393, 1913; 14, 81, 1913.

[2] H ion concentration may be expressed as gram of ionized H per liter of water. A neutral solution has a H ion concentration of 1×10^{-7}, or 0.000,000,1 gram per liter. It is customary to express the H ion concentration according to Sørensen's logarithmic notation. For example instead of expressing the H ion concentration of a neutral solution as 1×10^{-7} it is expressed as pH 7.00. An increasing H ion concentration decreases the pH and an increasing OH ion concentration increases the value. (See page 9.)

[3] Fisher: Nephritis, New York, 1912.

[4] Blatherwick: *Arch. Int. Med.*, 14, 409, 1914.

REACTION OF URINE AS INFLUENCED BY DIET[1]

Determination.	Basal diets[2]		1	2	3	4	5	6
	No. 1	No. 2	Baked potatoes (750 grams per day) + basal diet No. 1 (6 days)	Rice (210 grams per day) + basal diet No. 1 (4 days)	Cranberry sauce (300–600 grams per day) + basal diet, No. 1 (6 days)	Bread[3] (whole wheat) 450 grams for 1 day + basal diet, No. 1	Prunes (330–550 grams per day) + basal diet, No. 2 (3 days)	Cantaloup[4] (260 grams) per day) + basal diet, No. 2 (5 days)
	5 days	5 days						
pH	7.19	5.57	7.74	7.48–6.90 7.14	6.30–5.70 6.19	6.80 (Previous day 6.90)	5.30–4.80 5.07	5.30–7.38 6.70
Titratable acidity (c.c. N/10)	275	474	196–216 203	166–297 233	391–488 407	350 (Previous day 297)	570–540–578 563	466–250 328
Ammonia N (grams)	0.310	0.464	0.221–0.248 0.238	0.166–0.251 0.198	0.219–0.391 0.305	0.280 (Previous day 0.251)	0.602–0.729 0.654	0.513–0.220 0.310

4. Hydrogen Ion Concentration of the Urine as Influenced by Alkali and Acid Ingestion.—The ingestion of certain organic salts of the alkalis, *e.g.*, sodium citrate and sodium bicarbonate will cause a decrease in the hydrogen ion concentration of the urine. The ingestion of acids (either organic or inorganic) or acid salts, *e.g.*, sodium dihydrogen phosphate will increase the hydrogen ion concentration of the urine. The alkalis are much more effective in producing changes in reaction than

INFLUENCE OF INGESTED SODIUM BICARBONATE ON H ION CONCENTRATION OF URINE

Experiment Number	Sodium Bicarbonate, Grams	pH before Bicarbonate Ingestion	Time of Collection of Specimen of Urine and pH				
			11.00 A. M.	12.00 Noon	1.00 P. M.	2.00 P. M.	3.00 P. M.
1	4	7.40	8.30	7.48	7.48	7.40	5.85
2	8	5.40	8.50	8.30	6.50	6.50	7.40
3	12	5.30	8.70	8.70	8.70	8.70	8.70
4	8	7.40	8.50	8.70	8.50	8.50	8.50
5	8	5.85	.....	.....	8.70	8.70	8.30
6	8	6.70	7.48	8.70	8.50	8.70	8.50

[1] Tabulated from data reported by Blatherwick (*Arch. Int. Med.*, 14, 409, 1914) Experiments all made on the same subject (B).

[2] Basal diet No. 1 contained 100 grams Graham crackers, 25 grams butter, 400 c.c. whole milk ingested at each of the three daily meals. One apple and one soft boiled egg added at supper. In diet No. 2 whole wheat crackers were substituted for the Graham crackers.

[3] This day was preceded by $NaHCO_3$ ingestion for three days and by rice ingestion for four days.

[4] This diet followed immediately after the diet of prunes (see 5).

are the acids. The influence of ingested alkali (sodium bicarbonate) is shown in the foregoing table containing data submitted by Henderson and Palmer.[1]

Blatherwick[2] reports a decrease in ammonia nitrogen output from 0.256 gram to 0.072 gram, and an accompanying decreased acidity under the influence of bicarbonate ingestion (25 grams in two days).

The influence of ingested acid (benzoic) is shown in the following data reported by Blatherwick.[2]

INFLUENCE OF BENZOIC ACID INGESTION[3]

Day	Titratable acidity (c.c. N/10)	pH	Ammonia N (grams)
1	392	6.15	0.292
2	410	6.15	0.374
3	443	6.00	0.422
4	434	6.00	0.408
5	468	5.70	0.418

(For further discussion of dietary alterations of urine reaction see preceding experiment.)

Experiments.—(a) Influence of Alkali.—Ingest a uniform diet consisting of milk, crackers, butter, peanut butter and distilled water for a period of two days. During the next two days take the same diet and ingest 24 grams of sodium bicarbonate between meals (12 in A. M. and 12 in P. M.). Collect the urine in 24-hour periods and analyze it for titratable acidity, H ion concentration and ammonia. Compare your results with those shown in table on page 878.

If desired the bicarbonate may be given in one dose of 8–12 grams and the urine collected in hourly specimens for the next five hours and each specimen analyzed. Data from such experiments are shown in table on page 878.

(b) Influence of Acid.—Proceed as above except that 1 gram of benzoic acid (in capsule) is ingested before each meal of the experimental period.

The experiment may also be varied by ingesting 10 grams of sodium dihydrogen phosphate early in the day and collecting the urine in hourly fractions or in one 24-hour sample.

From your experiments what do you conclude as to the relative efficiency of acid and alkali in altering the reaction of the urine?

[1] Henderson and Palmer: *Jour. Biol. Chem.*, 14, 81, 1913.

[2] See p. 878.

[3] One gram of benzoic acid in a capsule before each meal. Basal diet No. 1 described on page 878 was used.

IX. EXPERIMENTS ON METABOLISM OF FASTING

The metabolism of a fasting man is entirely different from the metabolism of a well-nourished person. The collection and analysis of the urine during a short fast (three to seven days) will demonstrate many important facts. The following table, which contains data from fasting tests made in the author's laboratory,[1] illustrates some of the points in which fasting metabolism differs from normal metabolism:

Abstinence from food for a few days can in no way operate to the disadvantage of a normal person. In fact individuals affected with certain types of gastro-intestinal disorders are benefited by fasting.

METABOLISM IN FASTING

Day of period	Body weight, kg.	Total N grams	Ammonia N grams	Creatine N grams	Acidity. c.c. N/10 NaOH	P_2O_5 grams	Chloride grams, NaCl
Preliminary Feeding Period							
1–4	Av. 74.16	10.430	0.112	None	238.6	2.768	9.007
Fasting Period							
1	73.32	10.072	0.288	0.269	328.9	2.616	5.035
2	71 98	15.072	0.642	0.073	677.1	2.509	3.231
3	70.92	14.463	0.862	0.089	770.4	2.851	2.539
4	70.24	13.080	1.201	0.068	664.2	2.490	1.253
5	69.61	11.801	1.266	0.033	525.0	2.376	1.474
6	69.12	11.214	1.373	0.022	462.4	1.186	1.132
7	68.70	10.734	1.371	0.003	438.9	0.955	1.137

The *fasting treatment*[2] is also being used with success in cases of diabetes mellitus and in the treatment of obesity.[3]

In order to determine experimentally how the fasting metabolism differs from normal metabolism proceed as follows: Ingest an ordinary mixed diet and collect your urine (see page 789) **for a day. Measure the volume and analyze the sample for total nitrogen, ammonia, creatine, sodium chloride, total phosphates and acidity**[4] (for methods see Chapter XXXI). **For the next few days (three to**

[1] The chloride, phosphate and acidity determinations were collected during one seven-day fast and the other data collected during a different fast on the same man. (See Howe, Mattill and Hawk: *Jour. Amer. Chem. Soc.*, 33, 568, 1911; and Wilson and Hawk: *Jour. Amer. Chem. Soc.*, 36, 137, 1914.)

[2] Allen· *Amer. Jour. Med. Sci.*, 150, 480, 1915.

[3] Folin and Denis: *Jour. Biol. Chem.*, 21, 183, 1915.

[4] A more accurate experiment may be carried out by ingesting a uniform diet of known composition (see page 851) for a few days before the fast.

seven as desired) ingest nothing but water and collect the urine accurately and analyze for the constituents enumerated above. Tabulate your results and compare them with those given in the table above.

Student Exercise on Planning of Experimental Diets.—With the aid of the data given in the tables on pages 804, 805 and 850, and of other available reference books[1] from which the calorific value, nitrogen content, vitamin content, etc. may be obtained, plan experimental diets as described below, using the following typical foodstuffs: Casein, gelatin, gluten, whole wheat, lean beef heart, dried milk, starch, cane sugar,[2] lard, Crisco, butter, cod liver oil, yeast, salt mixtures (see pp. 801, 831 and 837) etc.

Plan diets which may be predicted to have the following effects, and hand in a report to your instructor explaining the reasons for your selections.

1. A diet which will *produce ophthalmia.*
2. A diet which will promote growth but *cause rickets.*
3. A diet which will *cure* or *prevent rickets.*
4. A diet which will *prevent growth* but not cause rickets.
5. A deit which will *restore growth* following Diet 4.
6. A diet which will *prevent normal reproduction.*
7. A diet which will *cause scurvy.*
8. A diet which will *cure* or *prevent scurvy.*
9. A diet which will *cause ketonuria.*
10. A diet which will correct Diet 9.
11. A diet which will *cause polyneuritis.*
12. A diet which will correct Diet 11.
13. A diet which will be *conducive to longevity* and the birth and rearing of *sturdy offspring.*
14. A diet which will *cause pellagra.*
15. A diet which will *cure pellagra.*
16. A diet which will *facilitate normal reproduction.*

[1] See note 1, p. 789. Also Sherman, "Food Products," 2nd Ed., Macmillan Co., 1925; and Sherman, "Chemistry of Food and Nutrition," 3rd Ed. Macmillan Co., 1926

[2] Too much sugar in the diet of dogs will cause diarrhea; starch is therefore largely used to supply carbohydrate.

Alizarin.—A 1 per cent solution of alizarin mono-sodium sulphonate in water.

Aluminum Hydroxide Cream.—To a 1 per cent solution of ammonium alum at room temperature add a slight excess of a 1 per cent solution of ammonium hydroxide. Wash by decantation until the wash water shows only the faintest trace of residue on evaporation.

Ammoniacal Silver Solution.—Dissolve 26 grams of silver nitrate in about 500 c.c. of water, add enough ammonium hydroxide to redissolve the precipitate which forms upon the first addition of the ammonium hydroxide and make the volume of the mixture up to 1 liter with water.

Ammonium Molybdate Solution.—(See Molybdate Solution, p. 887.)

Ammonium Thiocyanate Solution.—This solution is made of such a strength that 1 c.c. of it is equal to 1 c.c. of the standard silver nitrate solution mentioned below. To prepare the solution dissolve 12.9 grams of ammonium thiocyanate, NH_4SCN, in a little less than a liter of water. In a small flask place 20 c.c. of the standard silver nitrate solution, 5 c.c. of a cold saturated solution of ferric alum and 4 c.c. of nitric acid (sp. gr. 1.2), add water to make the total volume 100 c.c., and thoroughly mix the contents of the flask. Now run in the ammonium thiocyanate solution from a burette until a permanent *red-brown* tinge is produced. This is the end-reaction and indicates that the last trace of silver nitrate has been precipitated. Take the burette reading and calculate the amount of water necessary to use in diluting the ammonium thiocyanate in order that 10 c.c. of this solution may be exactly equal to 10 c.c. of the silver nitrate solution. Make the dilution and titrate again to be certain that the solution is of the proper strength.

Asbestos for Suction Filters.—The asbestos is shredded, placed in a wide mouth flask and covered with 10 per cent HCl. Heat on water-bath for five hours. Filter on Buchner funnel, wash free from acid, return to the flask, cover with 5 per cent NaOH and heat on water-bath for three hours. Filter, wash free from alkali, then with dilute acid and finally with water until free from acid. Suspend in a large volume of water, allow to settle for five minutes. Pour off the upper two-thirds and discard. Repeat the washing of the desired coarse portion several times until the supernatant liquid remains nearly clear.

Barfoed's Solution.—Dissolve 9 grams of neutral, crystallized copper acetate in 100 c.c. of water and add 1.2 c.c. of 50 per cent acetic acid.

Baryta Mixture.—A mixture consisting of 1 volume of a saturated solution of barium nitrate and 2 volumes of a saturated solution of barium hydroxide.

Basic Lead Acetate Solution.—This solution possesses the following formula:

Lead acetate	180 grams.
Lead oxide (Litharge)	110 grams.
Distilled water to make	1000 grams.

Dissolve the lead acetate in about 700 c.c. of distilled water, with boiling. Add this hot solution to the finely powdered lead oxide and boil for one-half hour with occasional stirring. Cool, filter and add sufficient distilled water to the filtrate to make the weight 1 kg.

Benedict's Solution.—Benedict has modified the Fehling solution and has succeeded in obtaining one which does not deteriorate upon long standing. It has the following composition:

Copper sulphate	17.3 grams.
Sodium citrate	173.0 grams.
Sodium carbonate	100.0 grams.
Distilled water to make 1 liter.	

With the aid of heat dissolve the sodium citrate and carbonate in about 800 c.c. of water. Pour (through a folded filter paper if necessary) into a glass graduate and make up to 850 c.c. Dissolve the copper sulphate in about 100 c.c. of water. Pour the carbonate-citrate solution into a large beaker or casserole and add the copper sulphate solution slowly, with constant stirring and make up to one liter. The mixed solution is ready for use and does not deteriorate upon long standing.

Benedict's Quantitative Sugar Reagent.

Copper sulphate (crystallized)	18.0 grams.
Sodium carbonate (crystallized, one-half the weight of the anhydrous salt may be used)	200.0 grams.
Sodium or potassium citrate	200.0 grams.
Potassium thiocyanate	125.0 grams.
Potassium ferrocyanide (5 per cent solution)	5.0 c.c.
Distilled water to make a total volume of	1000.0 c.c.

With the aid of heat dissolve the carbonate, citrate and thiocyanate in enough water to make about 800 c.c. of the mixture and filter if necessary.

Dissolve the copper sulphate separately in about 100 c.c. of water and pour the solution slowly into the other liquid, with constant stirring. Add the ferrocyanide solution, cool and dilute to exactly 1 liter. Of the

various constituents, the copper salt only need be weighed with exactness. Twenty-five c.c. of the reagent are reduced by 50 mg. of glucose.

Benedict's Sulphur Reagent.

Crystallized copper nitrate, sulphur-free or of known sulphur content	200 grams.
Sodium or potassium chlorate	50 grams.
Distilled water to	1000 c.c.

Bial's Reagent.

Orcinol	1.5 grams.
Concentrated HCl	500 grams.
Ferric chloride (10 per cent)	20–30 drops.

Biuret Reagent, Gies.—This reagent consists of 10 per cent KOH solution to which 25 c.c. of 3 per cent $CuSO_4$ solution per liter has been added. This imparts a slight though distinct blue color to the clear liquid.

Biuret Paper (Kantor and Gies).—Immerse filter paper in Gies' Biuret Reagent (above) then dry and cut into strips.

Boneblack, Purification of.—Treat 250 gms. of commercial boneblack with 1500 c.c. of 1: 4 HCl solution. Boil for half an hour. Filter the boneblack from the acid solution by means of a Buchner funnel and aspirating pump. Wash with hot water until the washings are neutral to litmus paper. Dry and powder.

Buffer Solutions, Standard.—See pages 13, 449 and 708.

Cleaning Solution.—To a few grams of sodium bichromate crystals dissolved in a minimum quantity of water, add about 500 c.c. of concentrated sulphuric acid (technical grade is satisfactory).

Cochineal Solution.—A saturated solution of cochineal in 30 per cent alcohol.

Combined Hydrochloric Acid (Protein Salt).—To prepare so-called *combined* hydrochloric acid simply add a soluble protein such as Witte's peptone to *free* hydrochloric acid of the desired strength until it no longer responds to free acid tests (see chapter on Gastric Digestion). For example, if 0.2 per cent combined acid is required the protein would be added to 0.2 per cent *free* hydrochloric acid.

Strictly speaking there is no such thing as "combined" acid in this sense. When the protein is added a *protein salt* of the acid is formed which ionizes differently from the free acid.

Congo Red.—Dissolve 0.5 gram of Congo red in 90 c.c. of water and add 10 c.c. of 95 per cent alcohol.

Congo Red-Fibrin.—This may be prepared by placing fibrin in faintly alkaline Congo red solution and heating to 80°C. The fibrin is then washed and preserved under glycerol.

Ehrlich's Diazo Reagent.—Two separate solutions should be prepared and mixed in definite proportions when needed for use.

(*a*) Five grams of sodium nitrite dissolved in 1 liter of distilled water.

(*b*) Five grams of sulphanilic acid and 50 c.c. of hydrochloric acid in 1 liter of distilled water.

Solutions (*a*) and (*b*) should be preserved in well-stoppered vessels and mixed in the proportion 1:50 when required. Green asserts that greater delicacy is secured by mixing the solutions in the proportion 1:100. The sodium nitrite deteriorates upon standing and becomes unfit for use in the course of a few weeks.

Esbach's Reagent.—Dissolve 10 grams of picric acid and 20 grams of citric acid in 1 liter of water.

Fehling's Solution.—Fehling's solution is composed of two definite solutions—a copper sulphate solution and an alkaline tartrate solution, which may be prepared as follows:

Copper sulphate solution = 34.65 grams of copper sulphate dissolved in water and made up to 500 c.c.

Alkaline tartrate solution = 125 grams of potassium hydroxide and 173 grams of Rochelle salt dissolved in water and made up to 500 c.c.

These solutions should be preserved separately in rubber-stoppered bottles and mixed in equal volumes when needed for use. This is done to prevent deterioration.

Folin-McEllroy Reagent.—Dissolve 100 g. of sodium pyrophosphate, 30 g. of disodium phosphate and 50 g. of dry sodium carbonate in approximately 1 liter of water by the aid of a little heat. Dissolve separately 13 g. of copper sulphate in about 200 c.c. of water. Pour the copper sulphate solution into the phosphate-carbonate solution and shake.

Furfural Solution.—Add 1 c.c. of furfural to 1000 c.c. of distilled water.

Fusion Mixture.—Two parts of sodium carbonate to one part of potassium nitrate.

Guaiac Solution.—Dissolve 0.5 gram of guaiac resin in 30 c.c. of 95 per cent alcohol.

Günzberg's Reagent.—Dissolve 2 grams of phloroglucinol and 1 gram of vanillin in 100 c.c. of 95 per cent alcohol.

Haines' Solution.—This solution may be prepared by dissolving 8.314 grams of copper sulphate in 400 c.c. of water adding 40 c.c. of glycerol and 500 c.c. of 5 per cent potassium hydroxide solution.

Hopkins-Cole Reagent.—To 1 liter of a saturated solution of oxalic acid add 60 grams of sodium amalgam and allow the mixture to stand until the evolution of gas ceases. Filter and dilute with 2–3 volumes of water.

Hopkin's-Cole Reagent (Benedict's Modification).—Ten grams of powdered magnesium are placed in a large Erlenmeyer flask and shaken up with enough distilled water to liberally cover the magnesium. Two hundred and fifty c.c. of a cold, saturated solution of oxalic acid is now added slowly. The reaction proceeds very rapidly and with the liberation of much heat, so that the flask should be cooled under running water during the addition of the acid. The contents of the flask are shaken after the addition of the last portion of the acid and then poured upon a filter, to remove the insoluble magnesium oxalate. A little wash water is poured through the filter, the filtrate acidified with acetic acid to prevent the partial precipitation of the magnesium on long standing, and made up to a liter with distilled water. This solution contains only the magnesium salt of glyoxylic acid.

Hydrochloric Acid Standard (N/10) Solution.—See pages 704 and 705.

Hypobromite Solution.—The ingredients of this solution should be prepared in the form of *two* separate solutions which may be united as needed.

(*a*) Dissolve 125 grams of sodium bromide in water, add 125 grams of bromine and make the total volume of the solution 1 liter.

(*b*) A solution of sodium hydroxide having a specific gravity of 1.25. This is approximately a 22.5 per cent solution.

Preserve both solutions in rubber-stoppered bottles and when needed for use mix 1 volume of solution (*a*), 1 volume of solution (*b*), and 3 volumes of water.

Iodine Solution (N/10).—Weigh out 12.685 grams of pure resublimed iodine into a small weighing bottle using a porcelain spatula. Dissolve 18 grams of pure KI in about 150 c.c. of water. Transfer the iodine to a liter flask washing out the last traces with some of the KI solution, which is then poured into the flask. Stopper and shake occasionally until dissolved. If necessary a few more crystals of KI may be added to aid solution. Dilute to the mark and mix well. Keep in glass-stoppered bottle in cool dark place. Standardize at once against N/10 sodium thiosulphate solution. Measure out accurately 25 c.c. of the iodine solution into an Erlenmeyer flask, run in sodium thiosulphate until the color is pale yellow, then add a few cubic centimeters of a 1 per cent solution of starch (preferably soluble starch) and titrate to disappearance of blue color. Care should be taken near the end point.

Iodine Solution.—Prepare a 2 per cent solution of potassium iodide and add sufficient iodine to color it a deep yellow.

Lead Acetate, Basic. (See Basic Lead Acetate.)

Lime Water.—Shake up an excess of calcium oxide or hydroxide with distilled water and leave well-stoppered over night. Decant clear supernatant solution and keep free from CO_2 of air.

Litmus-Milk Powder.—Add 1 part of powdered litmus to 50 parts of dried milk powder. To make a litmus milk *solution* add 1 part of this powder to 9 parts of water.

Lugol's Solution.—Dissolve 4 grams of iodine and 6 grams of potassium iodide in 100 c.c. of distilled water.

Magnesia Mixture.—Dissolve 175 grams of magnesium sulphate and 350 grams of ammonium chloride in 1400 c.c. of distilled water. Add 700 grams of concentrated ammonium hydroxide, mix thoroughly, and preserve the mixture in a glass-stoppered bottle.

Magnesium Nitrate Solution for Ignition.—Dissolve 320 grams of calcined magnesia in nitric acid, avoiding an excess of the latter; then add a little calcined magnesia in excess; boil; filter from the excess of magnesia, ferric oxide, etc., and dilute with water to 2 liters.

Methyl Red.—Saturated solution in 50 per cent alcohol.

Methyl Orange.—Dissolve 0.1 gm. of methyl orange in 100 c.c. of distilled water.

Millon's Reagent.—Digest 1 part (by weight) of mercury with 2 parts (by weight) of nitric acid (sp. gr. 1.42) and dilute the resulting solution with 2 volumes of water.

Molisch's Reagent.—A 15 per cent alcoholic solution of α-naphthol.

Molybdate Solution.—Dissolve 100 grams of molybdic acid in 144 c.c. of ammonium hydroxide (sp. gr. 0.90) and 271 c.c. of water; slowly and with constant stirring pour the solution thus obtained into 489 c.c. of nitric acid (sp. gr. 1.42) and 1148 c.c. of water. Keep the mixture in a warm place for several days, or until a portion heated to 40°C. deposits no yellow precipitate of ammonium phosphomolybdate. Decant the solution from any sediment and preserve in glass-stoppered bottles.

α-Naphthol Solution.—Dissolve 1 gram of α-naphthol in 100 c.c. of 95 per cent alcohol.

Nessler's Reagent.—(*a*) *Formula of Folin and Wu.* Nessler's solution is an alkaline solution of the double iodide of mercury and potassium ($HgI_2 \cdot 2KI$). Introduce into a 500 c.c. Florence flask 150 gm. potassium iodide and 110 gm. of iodine; add 100 c.c. of water and an excess of metallic mercury, 140–150 gm. Shake the flask continuously and vigorously for 7–15 minutes or until the dissolved iodine has nearly all disappeared. The solution becomes quite hot. When the red iodine solution has begun to become visibly pale, though still red, cool in running water and continue the shaking until the reddish color of the iodine has been replaced by the greenish color of the double iodide. The whole operation does not usually take more than 15 minutes. Decant the solution, washing mercury and flask with liberal quantities of distilled water. Dilute solution and washings

to 2 liters. If the cooling was begun in time the resulting reagent is clear enough for immediate dilution with 10 per cent alkali and water and the finished solution can be used at once for nesslerization. From this stock solution of potassium mercuric iodide prepare final Nessler's solutions as follows: Introduce into a flask of at least 5 liters capacity, 3500 c.c. of 10 per cent sodium hydroxide solution, add 750 c.c. of the double iodide solution and 750 c.c. of distilled water, making 5 liters of solution. The 10 per cent NaOH should be made from a saturated solution (containing about 55 gm. per 100 c.c.) which has been allowed to stand until the carbonate has settled, the clear solution being decanted and used. This solution should have been standardized with an accuracy of at least 5 per cent.

The alkalinity of the Nessler's reagent is important and may be checked by titrating with it 20 c.c. portions of N HCl. A good end point with phenolphthalein should be obtained at 11 to 11.5 c.c. If as low as 9.5 c.c. are required the solution is too alkaline. One c.c. of the dilute (1:1) acid digestion mixture should also require 9 to 9.3 c.c. of Nessler's solution to neutralize it.

(*b*) *Formula of Bock and Benedict.*—Place 100 gm. mercuric iodide and 70 gm. potassium iodide in a liter volumetric flask and add about 400 c.c. of water. Rotate until solution is complete. Now dissolve 100 gm. NaOH in about 500 c.c. of water, cool thoroughly and add with constant shaking to the mixture in the flask, then make up with water to the liter mark. This usually becomes perfectly clear. When the small amount of brownish red precipitate which forms, settles out the supernatant fluid is ready to be poured off and used.

Neutral Olive Oil.—Shake ordinary olive oil with a 10 per cent solution of sodium carbonate, extract the mixture with ether, and remove the ether by evaporation. The residue is *neutral* olive oil.

Neutral Red.—A 1 per cent solution in 50 per cent alcohol.

p-Nitrophenol.—A 1 per cent solution in 50 per cent alcohol.

Nylander's Reagent.—Digest 2 grams of bismuth subnitrate and 4 grams of Rochelle salt in 100 c.c. of a 10 per cent solution of potassium hydroxide. The reagent should then be cooled and filtered.

Obermayer's Reagent.—Add 2–4 grams of ferric chloride to a liter of hydrochloric acid (sp. gr. 1.19).

Oxalic Acid Standard (N/10) Solution.—See page 793.

Permutit.—A synthetic aluminium silicate obtained from the Permutit Company, New York. Only such preparations as have passed through a 60 mesh sieve and do not pass through an 80 mesh sieve should be used. It should give off very little dust or turbid material to water and settle in a few seconds. It may be used more

than once by washing first with water, then with 2 per cent acetic acid and finally with water again.

Phenolphthalein.—Dissolve 1 gram of phenolphthalein in 100 c.c. of 95 per cent alcohol.

Phenylhydrazine Mixture.—This mixture is prepared by combining 2 parts of phenylhydrazine-hydrochloride and 3 parts of sodium acetate *by weight*. These are thoroughly mixed in a mortar.

Phenylhydrazine-Acetate Solution.—This solution is prepared by mixing 1 volume of glacial acetic acid, 1 volume of water, and 2 volumes of phenylhydrazine (the base).

Picric Acid, Pure.—As purchased picric acid contains 10 per cent of added water. By exposure to the air between large filter papers (best in a warm place) the water will disappear by evaporation. Dry picric acid may be prepared in this way. If the alkaline picrate formed from this acid gives too deep a color, it may be purified as follows:

(*a*) *Method of Benedict.*—It is best to start with the "technical" grade of commercial picric acid, and there is no need for preliminary removal of the 10 per cent of water which such samples contain. Four hundred gm. of the moist picric acid are placed in a 2 liter Pyrex flask and 1 liter of pure benzene is added. The mixture is heated to vigorous boiling on an *electric plate* with occasional shaking at first to avoid bumping. Soon after the boiling point is reached the picric acid practically wholly dissolves, leaving a residue of dirt and foreign material, together with the water, which settle quickly to the bottom when the mixture is not boiling. The hot mixture (as nearly boiling as possible) is poured upon a large fluted filter which has been previously moistened with benzene, the solution being poured slowly enough so that most of the foreign sediment, together with the water, remains in the flask. This material (about 50 c.c. in volume) should be discarded, and not poured upon the filter. A hot water funnel is convenient for this filtration but can be dispensed with if necessary. If the hot water funnel is used the flame should be turned out just prior to beginning the filtration, since picric acid in benzene is a highly inflammable mixture. The clear filtrate should be received in a beaker of about 2 liters capacity. After the filtration is completed (it should not require more than 10 to 15 minutes) the beaker is covered with a large watch-glass, and heated on the electric plate until the picric acid which has begun to crystallize is again brought into solution. The covered beaker is then allowed to stand at room temperature for several hours or preferably over night without agitation. After this period the picric acid will be found to have crystallized on the bottom and sides of the beaker in large hard yellow-brown crystals, from which the excess of benzene can

readily be drained without recourse to any filtration. The crystalline mass is washed twice by gentle rotary shaking with 75 c.c. portions of benzene, and the residue finally allowed to drain thoroughly for 15 to 30 minutes. For the draining it is convenient to invert partially the beaker in a large glass funnel. The crystallized picric acid can now be freed from the last of the benzene by drying in the air (which requires a long time and is hence undesirable in the average laboratory on account of access of impurities) or better by placing in an air bath at about 80° for a few hours, with occasional stirring. The product should be finally powdered by *gentle* rubbing in a mortar, and preserved in a brown glass-stoppered bottle. About 85 per cent of the picric acid used is recovered in the purified form. The benzene may be recovered by distillation preferably *in vacuo* from a water bath.

(*b*) *Method of Folin and Doisy.*—Transfer about 600 gm. of wet picric acid, or about a pound of dry picric acid, to a large beaker (capacity not less than 4 liters). Pour on boiling water until the beaker is nearly full and add 200 c.c. of saturated (50 per cent) sodium hydroxide solution. Stir, and if necessary heat again until all the picric acid has been dissolved, yielding a deep red picrate solution. To the hot solution add rather slowly, with stirring, 200 gm. of sodium chloride. Cool in running water to about 30°C., with occasional stirring. Filter on a large Buchner funnel and wash a few times with 5 per cent sodium chloride solution. Transfer the picrate to a large beaker, fill with boiling water, and when the picrate is dissolved add, with stirring, first 50 c.c. of 10 per cent sodium hydroxide solution, and then 100 gm. of sodium chloride. Cool to 30°C., with stirring, filter, and wash with sodium chloride solution, as before. Repeat the solution and precipitation of the sodium picrate once more, but for the last washing of the last precipitated picrate use distilled water instead of the sodium chloride solution.

Dissolve the purified picrate in the same large beaker with boiling distilled water, and filter while hot on a large folded filter, collecting the filtrate in a large flask. To the hot filtrate add 100 c.c. of concentrated sulphuric acid, previously diluted with about two volumes of water. The liberated picric acid begins to come out at once. Put a beaker over the mouth of the flask and cool under running tap water to about 30°C. Filter with suction as before and wash free from sulphates with distilled water.

(*c*) *Method of Halverson and Bergeim.*—To 50 gm. of picric acid add 700 c.c. of distilled water. Boil until clear and while boiling add 10 c.c. of concentrated hydrochloric acid. Cool. Wash by decantation with 100 c.c. of distilled water. Repeat the recrystallization.

Transfer to a Buchner funnel and wash with about 150 c.c. of water. Dry in a desiccator or between filter papers.

Picric Acid, Saturated Solution.—This may be prepared either by allowing distilled water to stand in contact with an excess of picric acid with occasional shaking, or by making a 1.2 per cent solution.

Potassium Permanganate Standard (N/10) Solution.—Dissolve 3.162 grams of pure potassium permanganate in a liter of distilled water, allow to stand a few days, and filter through glass wool. Standardize against N/10 oxalic acid solution or against pure dry sodium or potassium oxalate. One c.c. of N/10 permanganate is equivalent to 7.0 mg. of sodium oxalate.

Roberts' Reagent.—Mix 1 volume of concentrated nitric acid and 5 volumes of a saturated solution of magnesium sulphate.

Sahli's Reagent.—This reagent consists of a mixture of equal parts of a 48 per cent solution of potassium iodide and an 8 per cent solution of potassium iodate.

Schweitzer's Reagent.—Add potassium hydroxide to a 5 per cent solution of copper sulphate which contains 5 per cent ammonium chloride. Filter off the precipitate of cupric hydroxide, wash it, and bring 3 grams of the moist cupric hydroxide into solution in a liter of 20 per cent ammonium hydroxide.

Seliwanoff's Reagent.—Dissolve 0.05 gram of resorcinol in 100 c.c. of dilute (1:2) hydrochloric acid.

Sodium Acetate Solution.—Dissolve 100 grams of sodium acetate in 800 c.c. of distilled water, add 100 c.c. of 30 per cent acetic acid to the solution, and make the volume of the mixture up to 1 liter with distilled water.

Sodium Alcoholate (N/10) Solution.—The sodium alcoholate is made by dissolving 2.3 grams of cleaned metallic sodium in 1 liter of absolute alcohol. It is advisable that it be slightly weaker than stronger than tenth-normal. It may be standardized against pure benzoic acid in washed chloroform. It may also be standardized against N/10 HCl provided the alcoholate solution contains not more than traces of carbonate.

Sodium Alizarin Sulphonate.—Dissolve 1 gram of sodium alizarin sulphonate in 100 c.c. of water.

Sodium Hydroxide Saturated Solution.—Shake up about 110 gm. of best quality NaOH with 100 c.c. of distilled water in a 300 c.c. Erlenmeyer flask (Pyrex). Stopper and allow to stand for a couple of days or until the sodium carbonate settles to the bottom leaving a clear solution of NaOH practically free from carbonate, and containing about 75 gm. NaOH per 100 c.c.

Sodium Hydroxide Standard (N/10) Solution.—See pages 703 and 704.

Sodium Thiosulphate Standard (N/10) Solution.—Weigh out 25 grams of ordinary c.p. sodium thiosulphate or 24.83 grams of the pure dry recrystallized salt. Dissolve in water and dilute to a liter. Boiled distilled water must be used. Keep in a bottle with a siphon arrangement and carrying a soda lime tube to exclude CO_2.

It is best standardized against acid potassium iodate $KH(IO_3)_2$. Weigh out accurately 0.3249 gram of acid potassium iodate. Dissolve in 50 c.c. of water, heating gently if necessary. Transfer the solution to a 100 c.c. flask, rinsing the beaker carefully and make to mark with water. This solution is exactly decinormal. Pipette out 25 c.c. into an Erlenmeyer flask, add 1 gram of potassium iodide dissolved in a little water, and a few cubic centimeters of dilute hydrochloric acid. Titrate immediately with the thiosulphate solution. When the solution becomes pale yellow add a few cubic centimeters of 1 per cent solution of soluble starch and titrate to loss of blue color.

Sodium Tungstate Solution.—A 10 per cent solution of sodium tungstate in water. The c.p. sodium tungstate made by the J. T. Baker Chemical Co., Phillipsburg, N. J., is satisfactory. If not easily soluble prepare a hot 10 per cent solution, cool, and titrate 25 c.c. with 10 per cent NaOH to a pink color with phenolphthalein which lasts for 3 minutes. Add a proportional amount of NaOH to the main solution.

Starch Paste.—Grind 2 grams of starch powder in a mortar with a small amount of water. Bring 200 c.c. of water to the boiling-point and add the starch mixture from the mortar with continuous stirring. Bring again to the boiling-point and allow it to cool. This makes an approximate 1 per cent starch paste which is a very satisfactory strength for general use.

Stokes' Reagent.—A solution containing 2 per cent ferrous sulphate and 3 per cent tartaric acid. When needed for use a small amount should be placed in a test-tube and ammonium hydroxide added until the precipitate which forms on the first addition of the hydroxide has entirely dissolved. This produces *ammonium ferrotartrate*, which is a reducing agent.

Sulphuric Acid, N/12.—Add 2.5 c.c. concentrated sulphuric acid to a liter of distilled water. Standardize against alkali of known strength. (See page 368.)

Sulphuric Acid, Two-thirds Normal.—Add 35 gm. of concentrated c.p. sulphuric acid to a liter of distilled water. Standardize against alkali of known strength.

Tanret's Reagent.—Dissolve 1.35 grams of mercuric chloride in 25 c.c. of water, add to this solution 3.32 grams of potassium iodide

dissolved in 25 c.c. of water, then make the total solution up to 60 c.c. with distilled water and add 20 c.c of glacial acetic acid to the mixture.

Tincture of Iodine.—Dissolve 70 grams of iodine and 50 grams of potassium iodide in 1 liter of 95 per cent alcohol.

Töpfer's Reagent.—Dissolve 0.5 gram of di-methylaminoazobenzene in 100 c.c. of 95 per cent alcohol.

Tropæolin OO.—Dissolve 0.05 gram of tropæolin OO in 100 c.c. of 50 per cent alcohol.

Uffelmann's Reagent.—Add a 5 per cent solution of ferric chloride to a 1 per cent solution of carbolic acid until an amethyst-blue color is obtained.

Uranium Acetate Solution.—Dissolve about 35.0 grams of uranium acetate in 1 liter of water with the aid of heat and 3–4 c.c. of glacial acetic acid. Let stand a few days and filter. Standardize against a phosphate solution containing 0.005 gram of P_2O_5 per cubic centimeter. For this purpose dissolve 14.721 grams of pure air-dry sodium ammonium phosphate ($NaNH_4HPO_4+4H_2O$) in water to make a liter. To 20 c.c. of this phosphate solution in a 200 c.c. beaker add 30 c.c. of water and 5 c.c. of sodium acetate solution (see p. 891) and titrate with the uranium solution to the correct end reaction as indicated in the method proper, page 777. If exactly 20 c.c. of uranium solution are required 1 c.c. of the solution is equivalent to 0.005 gram P_2O_5. If stronger than this dilute accordingly and check again by titration.

APPENDIX

TABLE 1

MILLIMETERS TO BE SUBTRACTED FROM BAROMETER (BRASS SCALE) READINGS TO REDUCE THEM TO 0°C.[1]

Temp. °C.	Barometric pressure in millimeters				
	740	750	760	770	780
15.0	1.81	1.83	1.86	1.88	1.91
15.5	1.87	1.89	1.92	1.94	1.97
16.0	1.93	1.96	1.98	2.01	2.03
16.5	1.99	2.02	2.04	2.07	2.09
17.0	2.05	2.08	2.10	2.13	2.16
17.5	2.11	2.14	2.16	2.19	2.22
18.0	2.17	2.20	2.23	2.26	2.29
18.5	2.23	2.26	2.29	2.32	2.35
19.0	2.29	2.32	2.35	2.38	2.41
19.5	2.35	2.38	2.41	2.45	2.48
20.0	2.41	2.44	2.47	2.51	2.54
20.5	2.47	2.50	2.54	2.57	2.61
21.0	2.53	2.56	2.60	2.63	2.67
21.5	2.59	2.63	2.66	2.70	2.73
22.0	2.65	2.69	2.72	2.76	2.79
22.5	2.71	2.75	2.78	2.82	2.86
23.0	2.77	2.81	2.84	2.88	2.92
23.5	2.83	2.87	2.91	2.95	2.99
24.0	2.89	2.93	2.97	3.01	3.05
24.5	2.95	2.99	3.03	3.07	3.11
25.0	3.01	3.05	3.09	3.13	3.17
25.5	3.07	3.11	3.15	3.20	3.24
26.0	3.13	3.17	3.21	3.26	3.30
26.5	3.19	3.23	3.28	3.32	3.36
27.0	3.25	3.29	3.34	3.38	3.42
27.5	3.31	3.35	3.40	3.45	3.49
28.0	3.37	3.41	3.46	3.51	3.55
28.5	3.43	3.48	3.52	3.57	3.62
29.0	3.49	3.54	3.58	3.63	3.68
29.5	3.55	3.60	3.65	3.69	3.74
30.0	3.61	3.66	3.71	3.75	3.80

[1] Landolt-Börnstein: Physikalisch-chemische Tabellen, 35, 1905.

TABLE 2

ANALYSIS OF THE OXIDATION OF MIXTURES OF CARBOHYDRATE AND FAT

Table of Zuntz and Schumburg modified by Lusk[1]

R.Q.	Percentage of total oxygen consumed		Percentage of total heat produced		Calories per liter O_2		
	Carbohydrate	Fat	Carbohydrate	Fat	Number	Log.	Log. + log. of 60
0.707	0	100.00	0	100.00	4.686	.67080	.44895
.71	1.02	99.00	1.10	98.90	4.690	.67114	.44929
.72	4.44	95.60	4.76	95.20	4.702	.67228	.45043
.73	7.85	92.20	8.40	91.60	4.714	.67342	.45157
.74	11.30	88.70	12.00	88.00	4.727	.67456	.45271
.75	14.70	85.30	15.60	84.40	4.739	.67569	.45384
.76	18.10	81.90	19.20	80.80	4.751	.67682	.45497
.77	21.50	78.50	22.80	77.20	4.764	.67794	.45609
.78	24.90	75.10	26.30	73.70	4.776	.67906	.45721
.79	28.30	71.70	29.90	70.10	4.788	.68018	.45833
.80	31.70	68.30	33.40	66.60	4.801	.68129	.45944
.81	35.20	64.80	36.90	63.10	4.813	.68241	.46056
.82	38.60	61.40	40.30	59.70	4.825	.68352	.46167
.83	42.00	58.00	43.80	56.20	4.838	.68463	.46278
.84	45.40	54.60	47.20	52.80	4.850	.68573	.46388
.85	48.80	51.20	50.70	49.30	4.862	.68683	.46498
.86	52.20	47.80	54.10	45.90	4.875	.68793	.46608
.87	55.60	44.40	57.50	42.50	4.887	.68903	.46718
.88	59.00	41.00	60.80	39.20	4.899	.69012	.46827
.89	62.50	37.50	64.20	35.80	4.911	.69121	.46936
.90	65.90	34.10	67.50	32.50	4.924	.69230	.47045
.91	69.30	30.70	70.80	29.20	4.936	.69339	.47154
.92	72.70	27.30	74.10	25.90	4.948	.69447	.47262
.93	76.10	23.90	77.40	22.60	4.961	.69555	.47370
.94	79.50	20.50	80.70	19.30	4.973	.69663	.47478
.95	82.90	17.10	84.00	16.00	4.985	.69770	.47585
.96	86.30	13.70	87.20	12.80	4.998	.69877	.47692
.97	89.80	10.20	90.40	9.58	5.010	.69984	.47799
.98	93.20	6.83	93.60	6.37	5.022	.70091	.47906
.99	96.60	3.41	96.80	3.18	5.035	.70197	.48012
1.00	100.00	0	100.00	0	5.047	.70303	.48118

The last column has been added to facilitate the expression of oxygen absorbed per minute in terms of calories per hour.

[1] Lusk: *Jour. Biol. Chem.*, 59, 42, 1924.

TABLE 3 [1]

CALORIES PER SQUARE METER OF BODY SURFACE PER HOUR

(Du Bois height-weight formula)

Age, years	Males	Females	Age, years	Males	Females
4	56	53	39	39.0	36.4
5	55	52	40	38.9	36.3
6	54	51	41	38.9	36.2
7	53	50	42	38.8	36.2
8	52	49	43	38.8	36.1
9	51	48	44	38.7	36.1
10	50	47	45	38.6	36.0
11	49	46	46	38.5	36.0
12	48	45	47	38.4	35.9
13	47	44	48	38.4	35.8
14	46	43	49	38.3	35.7
15	45	42	50	38.2	35.6
16	44	41	51	38.2	35.5
17	43	40	52	38.1	35.4
18	42	39	53	38.0	35.3
19	41	38	54	38.0	35.3
20	40	37	55	38.0	35.2
21 to 30	39.9	37	56	38.0	35.1
			57	37.9	35.0
31	39.8	36.8	58	37.9	35.0
32	39.7	36.7	59	37.8	35.0
33	39.5	36.7	60	37.7	34.9
34	39.3	36.6	61	37.7	34.9
35	39.2	36.5	62	37.6	34.8
36	39.1	36.5	63	37.5	34.8
37	39.1	36.5	64	37.4	34.7
38	39.0	36.4	65	37.3	34.7

[1] Basal metabolism in apparently normal *patients* undergoing a general physical examination. The averages are based on 254 boys and 220 girls between the ages of 4 and 20; and on 260 adult males and 378 adult females. The subjects were tested under the standard conditions for out-patients in the Respiration Laboratory of the New York Post Graduate Hospital. The results are probably not "basal," but are normal for the standard conditions of the test. (Bailey.)

TABLE 4

LOGARITHMS FOR THE REDUCTION OF SATURATED VOLUMES TO 0°C. DRY AND 760 mm. PRESSURE

Temp. °C.	Barometric pressure in millimeters (corrected for brass scale)									
	741	742	743	744	745	746	747	748	749	750
15.0	9.95817	.95876	.95935	.95995	.96055	.96114	.96173	.96232	.96292	.96351
15.5	.95715	.95774	.95834	.95894	.95954	.96013	.96072	.96131	.96191	.96249
16.0	.95614	.95673	.95733	.95793	.95853	.95912	.95971	.96030	.96090	.96149
16.5	.95514	.95573	.95633	.95693	.95753	.95812	.95871	.95930	.95990	.96049
17.0	.95410	.95470	.95530	.95590	.95650	.95709	.95768	.95827	.95887	.95946
17.5	.95307	.95367	.95427	.95487	.95547	.95606	.95665	.95724	.95784	.95843
18.0	.95204	.95264	.95324	.95384	.95444	.95503	.95562	.95622	.95682	.95741
18.5	.95101	.95161	.95221	.95281	.95340	.95399	.95458	.95518	.95578	.95637
19.0	.94996	.95056	.95116	.95176	.95236	.95295	.95354	.95413	.95473	.95532
19.5	.94889	.94949	.95009	.95069	.95129	.95188	.95247	.95307	.95367	.95426
20.0	94783	94843	94903	94963	95023	95082	95141	95201	95261	95320
20.5	94676	94736	94796	94856	94916	94975	95034	95094	95154	95214
21.0	94568	94628	94688	94748	94808	94867	94927	94987	95047	95106
21.5	94460	94520	94580	94640	94700	94759	94819	94879	94939	94998
22.0	94350	94410	94470	94530	94590	94649	94709	94769	94829	94889
22.5	94240	94300	94360	94420	94480	94539	94599	94659	94719	94779
23.0	94127	94187	94247	94307	94368	94428	94488	94548	94607	94666
23.5	94014	94074	94134	94195	94256	94316	94376	94436	94495	94555
24.0	93901	93961	94021	94081	94142	94202	94262	94322	94382	94442
24.5	93787	93847	93907	93967	94028	94088	94148	94208	94268	94328
25.0	93671	93731	93791	93852	93913	93973	94033	94093	94153	94213
25.5	93556	93616	93676	93737	93798	93858	93918	93978	94038	94098
26.0	93437	93497	93558	93619	93680	93740	93800	93860	93921	93980
26.5	93319	93379	93440	93501	93562	93622	93682	93742	93802	93862
27.0	93199	93259	93320	93381	93442	93502	93562	93622	93683	93744
27.5	93077	93138	93199	93260	93321	93381	93441	93501	93562	93622
28.0	92956	93017	93078	93139	93200	93260	93320	93380	93441	93501
28.5	92833	92893	92954	93014	93075	93135	93195	93256	93317	93379
29.0	92708	92769	92830	92891	92952	93012	93073	93134	93195	93255
29.5	92582	92643	92704	92765	92826	92886	92946	93007	93068	93129
30.0	92455	92516	92577	92638	92699	92759	92820	92881	92942	93002

TABLE 4 (*Continued*)

LOGARITHMS FOR THE REDUCTION OF SATURATED VOLUMES TO 0°C. DRY AND 760 mm. PRESSURE

Temp. °C.	Barometric pressure in millimeters (corrected for brass scale)									
	751	752	753	754	755	756	757	758	759	760
15.0	9.96409	.96468	.96527	.96586	.96644	.96703	.96761	.96819	.96878	.96936
15.5	96308	96366	96425	96484	96543	96601	96659	96718	96777	96835
16.0	96207	96266	96325	96384	96443	96501	96559	96618	96677	96735
16.5	96107	96166	96225	96284	96343	96401	96459	96518	96577	96635
17.0	96005	96064	96123	96182	96240	96299	96357	96416	96474	96532
17.5	95902	95961	96020	96079	96138	96197	96255	96314	96372	96430
18.0	95800	95859	95918	95977	96035	96094	96152	96211	96270	96328
18.5	95696	95755	95814	95873	95932	95990	96048	96107	96166	96225
19.0	95591	95650	95709	95768	95827	95885	95944	96003	96062	96120
19.5	95485	95544	95603	95662	95721	95780	95838	95897	95956	96014
20.0	95379	95438	95497	95556	95616	95674	95733	95792	95851	95909
20.5	95273	95332	95391	95450	95509	95567	95626	95685	95744	95803
21.0	95165	95224	95283	95342	95402	95460	95519	95578	95637	95696
21.5	95057	95116	95175	95234	95294	95353	95412	95471	95530	95588
22.0	94948	95007	95066	95125	95185	95244	95303	95362	95421	95479
22.5	94838	94897	94956	95015	95075	95134	95193	95252	95311	95370
23.0	94725	94784	94843	94903	94963	95022	95081	95140	95199	95258
23.5	94615	94674	94733	94793	94852	94912	94971	95030	95088	95147
24.0	94501	94560	94619	94679	94739	94798	94857	94916	94976	95034
24.5	94387	94446	94506	94566	94626	94685	94744	94803	94862	94921
25.0	94272	94331	94391	94451	94511	94570	94629	94688	94748	94807
25.5	94157	94216	94276	94336	94396	94455	94514	94573	94633	94692
26.0	94039	94099	94159	94219	94279	94338	94397	94456	94516	94575
26.5	93922	93982	94042	94102	94161	94220	94279	94339	94399	94458
27.0	93803	93863	93923	93983	94043	94102	94161	94220	94280	94340
27.5	93683	93743	93803	93863	93922	93982	94041	94101	94160	94219
28.0	93561	93621	93681	93741	93801	93860	93919	93979	94039	94099
28.5	93439	93499	93559	93619	93679	93738	93798	93858	93918	93977
29.0	93316	93376	93436	93496	93556	93615	93675	93735	93795	93855
29.5	93190	93250	93310	93370	93430	93490	93550	93610	93670	93729
30.0	93063	93123	93183	93244	93305	93364	93324	93484	93544	93604

TABLE 4 (*Continued*)
LOGARITHMS FOR THE REDUCTION OF SATURATED VOLUMES TO 0°C. DRY AND 760 mm. PRESSURE

Temp. °C.	Barometric pressure in millimeters (corrected for brass scale)									
	761	762	763	764	765	766	767	768	769	770
15.0	9.96994	.97052	.97110	.97168	.97226	.97283	.97340	.97398	.97456	.97513
15.5	96893	96951	97009	97067	97125	97182	97239	97297	97355	97413
16.0	96793	96851	96909	96967	97025	97083	97141	97198	97255	97313
16.5	96693	96751	96809	96867	96925	96982	97040	97098	97156	97213
17.0	96590	96648	96706	96764	96823	96880	96937	96995	97053	97111
17.5	96488	96546	96604	96662	96721	96778	96836	96894	96952	97009
18.0	96386	96444	96502	96560	96618	96676	96734	96792	96850	96907
18.5	96283	96341	96399	96457	96515	96573	96631	96689	96747	96804
19.0	96178	96236	96294	96352	96411	96469	96527	96585	96643	96700
19.5	96072	96130	96188	96246	96305	96363	96421	96479	96537	96594
20.0	95967	96025	96083	96142	96201	96258	96316	96374	96432	96490
20.5	95861	95919	95977	96036	96095	96152	96210	96268	96326	96384
21.0	95754	95812	95870	95929	95987	96046	96104	96162	96220	96277
21.5	95646	95704	95763	95822	95881	95939	95997	96055	96113	96171
22.0	95537	95595	95654	95713	95772	95830	95888	95946	96004	96062
22.5	95428	95486	95544	95603	95662	95720	95778	95836	95895	95953
23.0	95317	95375	95434	95493	95552	95610	95668	95726	95785	95843
23.5	95205	95263	95322	95381	95440	95499	95557	95615	95673	95731
24.0	95093	95152	95211	95270	95329	95387	95445	95503	95562	95620
24.5	94979	95038	95097	95156	95215	95273	95331	95389	95448	95507
25.0	94866	94925	94984	95043	95102	95160	95218	95276	95335	95394
25.5	94750	94809	94868	94927	94986	95044	95102	95161	95220	95278
26.0	94634	94693	94752	94811	94870	94928	94986	95045	95104	95162
26.5	94517	94576	94635	94694	94753	94812	94870	94929	94987	95045
27.0	94400	94459	94518	94577	94636	94694	94753	94811	94870	94929
27.5	94278	94337	94396	94455	94515	94573	94631	94690	94749	94808
28.0	94158	94217	94276	94335	94395	94453	94512	94571	94630	94688
28.5	94037	94096	94155	94214	94274	94333	94392	94450	94509	94568
29.0	93914	93973	94032	94091	94151	94210	94269	94328	94387	94445
29.5	93789	93848	93907	93967	94027	94086	94145	94204	94263	94322
30.0	93663	93722	93781	93841	93901	93960	94019	94078	94137	94196

TABLE 4 (*Concluded*)
LOGARITHMS FOR THE REDUCTION OF SATURATED VOLUMES TO 0°C. DRY AND 760 mm. PRESSURE

Temp. °C.	Barometric pressure in millimeters (corrected for brass scale)									
	771	772	773	774	775	776	777	778	779	780
15.0	9.97570	.97627	.97684	.97741	.97799	.97856	.97913	.97970	.98026	.98083
15.5	97470	97527	97584	97641	97698	97755	97812	97869	97926	97983
16.0	97370	97427	97484	97541	97599	97656	97713	97770	97826	97883
16.5	97270	97327	97384	97442	97500	97557	97614	97671	97727	97784
17.0	97168	97225	97282	97339	97397	97454	97511	97568	97625	97682
17.5	97066	97123	97180	97238	97296	97353	97410	97467	97524	97580
18.0	96964	97021	97078	97136	97194	97251	97308	97365	97422	97479
18.5	96861	96918	96975	97033	97091	97148	97205	97262	97319	97376
19.0	96757	96814	96872	96930	96988	97045	97102	97159	97216	97273
19.5	96651	96708	96766	96824	96882	96939	96996	97053	97111	97167
20.0	96547	96604	96662	96720	96778	96835	96892	96949	97006	97063
20.5	96441	96498	96556	96614	96672	96729	96786	96843	96901	96958
21.0	96335	96392	96450	96508	96565	96623	96680	96737	96794	96851
21.5	96228	96286	96344	96402	96459	96516	96573	96630	96688	96745
22.0	96119	96177	96235	96293	96351	96408	96465	96522	96580	96637
22.5	96011	96068	96126	96184	96241	96298	96355	96413	96471	96528
23.0	95901	95959	96017	96075	96132	96189	96246	96303	96361	96419
23.5	95789	95847	95905	95963	96020	96077	96134	96192	96250	96307
24.0	95678	95736	95794	95852	95910	95967	96024	96082	96140	96197
24.5	95565	95623	95681	95739	95796	95853	95911	95969	96027	96084
25.0	95452	95510	95568	95626	95684	95741	95799	95857	95914	95972
25.5	95336	95394	95452	95510	95568	95626	95684	95742	95799	95857
26.0	95220	95278	95336	95394	95453	95511	95569	95627	95684	95741
26.5	95103	95161	95219	95277	95336	95394	95452	95510	95567	95625
27.0	94987	95045	95103	95161	95220	95278	95336	95394	95451	95509
27.5	94866	94924	94982	95040	95099	95157	95215	95273	95331	95389
28.0	94746	94804	94862	94921	94980	95038	95096	95154	95212	95269
28.5	94626	94684	94742	94801	94860	94918	94976	95034	95092	95150
29.0	94503	94561	94620	94679	94738	94796	94854	94912	94970	95028
29.5	94380	94438	94496	94555	94614	94672	94730	94788	94847	94905
30.0	94254	94312	94371	94430	94489	94547	94605	94663	94722	94780

TABLE 5

VOLUME OF OXYGEN IN INSPIRED AIR CORRESPONDING TO THE NITROGEN IN 100 VOLUMES OF EXPIRED AIR

CO_2+O_2	.00	.01	.02	.03	.04	.05	.06	.07	.08	.09
19.5	21.33	21.33	21.32	21.32	21.32	21.32	21.31	21.31	21.31	21.30
19.6	21.30	21.30	21.30	21.30	21.29	21.29	21.29	21.28	21.28	21.28
19.7	21.28	21.27	21.27	21.27	21.27	21.26	21.26	21.26	21.26	21.25
19.8	21.25	21.25	21.24	21.24	21.24	21.24	21.23	21.23	21.23	21.23
19.9	21.22	21.22	21.22	21.22	21.21	21.21	21.21	21.20	21.20	21.20
20.0	21.20	21.19	21.19	21.19	21.19	21.18	21.18	21.18	21.18	21.17
20.1	21.17	21.17	21.17	21.16	21.16	21.16	21.15	21.15	21.15	21.15
20.2	21.14	21.14	21.14	21.14	21.13	21.13	21.13	21.13	21.12	21.12
20.3	21.12	21.11	21.11	21.11	21.11	21.10	21.10	21.10	21.10	21.09
20.4	21.09	21.09	21.09	21.08	21.08	21.08	21.08	21.07	21.07	21.07
20.5	21.07	21.06	21.06	21.06	21.05	21.05	21.05	21.05	21.04	21.04
20.6	21.04	21.04	21.03	21.03	21.03	21.02	21.02	21.02	21.02	21.01
20.7	21.01	21.01	21.01	21.00	21.00	21.00	21.00	20.99	20.99	20.99
20.8	20.99	20.98	20.98	20.98	20.97	20.97	20.97	20.97	20.96	20.96
20.9	20.96	20.96	20.95	20.95	20.95	20.95	20.94	20.94	20.94	20.93
21.0	20.93	20.93	20.93	20.92	20.92	20.92	20.92	20.91	20.91	20.91
21.1	20.91	20.90	20.90	20.90	20.89	20.89	20.89	20.89	20.88	20.88
21.2	20.88	20.88	20.87	20.87	20.87	20.87	20.86	20.86	20.86	20.86
21.3	20.85	20.85	20.85	20.84	20.84	20.84	20.84	20.83	20.83	20.83
21 4	20.83	20.82	20.82	20.82	20.82	20.81	20.81	20.81	20.80	20.80

TABLE 6
SO-CALLED SAGE NORMAL STANDARDS. (AUB AND DuBOIS)
Calories per square meter of body surface per hour
(Height-weight formula)

Age, years	Males	Females
14 to 16	46.0	43.0
16 to 18	43.0	40.0
18 to 20	41.0	38.0
20 to 30	39.5	37.0
30 to 40	39.5	36.5
40 to 50	38.5	36.0
50 to 60	37.5	35.0
60 to 70	36.5	34.0
70 to 80	35.5	33.0

TABLE 7
VAPOR TENSION OF WATER

°C	mm. Hg.	°C	mm. Hg.
10	9.1	20	17.4
11	9.8	21	18.5
12	10.4	22	19.6
13	11.1	23	20.9
14	11.9	24	22.2
15	12.7	25	23.5
16	13.5	26	25.0
17	14.4	27	26.5
18	15.3	28	28.1
19	16.3	29	29.7
		30	31.5

LOGARITHMS OF NUMBERS

Natural numbers	0	1	2	3	4	5	6	7	8	9	Proportional parts							
											1	2	3	4	5	6	7	8	9
10	0000	0043	0086	0128	0170	0212	0253	0294	0334	0374	4	8	12	17	21	25	29	33	37
11	0414	0453	0492	0531	0569	0607	0645	0682	0719	0755	4	8	11	15	19	23	26	30	34
12	0792	0828	0864	0899	0934	0969	1004	1038	1072	1106	3	7	10	14	17	21	24	28	31
13	1139	1173	1206	1239	1271	1303	1335	1367	1399	1430	3	6	10	13	16	19	23	26	29
14	1461	1492	1523	1553	1584	1614	1644	1673	1703	1732	3	6	9	12	15	18	21	24	27
15	1761	1790	1818	1847	1875	1903	1931	1959	1987	2014	3	6	8	11	14	17	20	22	25
16	2041	2068	2095	2122	2148	2175	2201	2227	2253	2279	3	5	8	11	13	16	18	21	24
17	2304	2330	2355	2380	2405	2430	2455	2480	2504	2529	2	5	7	10	12	15	17	20	22
18	2553	2577	2601	2625	2648	2672	2695	2718	2742	2765	2	5	7	9	12	14	16	19	21
19	2788	2810	2833	2856	2878	2900	2923	2945	2967	2989	2	4	7	9	11	13	16	18	20
20	3010	3032	3054	3075	3096	3118	3139	3160	3181	3201	2	4	6	8	11	13	15	17	19
21	3222	3243	3263	3284	3304	3324	3345	3365	3385	3404	2	4	6	8	10	12	14	16	18
22	3424	3444	3464	3483	3502	3522	3541	3560	3579	3598	2	4	6	8	10	12	14	15	17
23	3617	3636	3655	3674	3692	3711	3729	3747	3766	3784	2	4	6	7	9	11	13	15	17
24	3802	3820	3838	3856	3874	3892	3909	3927	3945	3962	2	4	5	7	9	11	12	14	16
25	3979	3997	4014	4031	4048	4065	4082	4099	4116	4133	2	3	5	7	9	10	12	14	15
26	4150	4166	4183	4200	4216	4232	4249	4265	4281	4298	2	3	5	7	8	10	11	13	15
27	4314	4330	4346	4362	4378	4393	4409	4425	4440	4456	2	3	5	6	8	9	11	13	14
28	4472	4487	4502	4518	4533	4548	4564	4579	4594	4609	2	3	5	6	8	9	11	12	14
29	4624	4639	4654	4669	4683	4698	4713	4728	4742	4757	1	3	4	6	7	9	10	12	13
30	4771	4786	4800	4814	4829	4843	4857	4871	4886	4900	1	3	4	6	7	9	10	11	13
31	4914	4928	4942	4955	4969	4983	4997	5011	5024	5038	1	3	4	6	7	8	10	11	12
32	5052	5065	5079	5092	5105	5119	5132	5145	5159	5172	1	3	4	5	7	8	9	11	12
33	5185	5198	5211	5224	5237	5250	5263	5276	5289	5302	1	3	4	5	6	8	9	10	12
34	5315	5328	5340	5353	5366	5378	5391	5403	5416	5428	1	3	4	5	6	8	9	10	11
35	5441	5453	5465	5478	5490	5502	5514	5527	5539	5551	1	2	4	5	6	7	9	10	11
36	5563	5575	5587	5599	5611	5623	5635	5647	5658	5670	1	2	4	5	6	7	8	10	11
37	5682	5694	5705	5717	5729	5740	5752	5763	5775	5786	1	2	3	5	6	7	8	9	10
38	5798	5809	5821	5832	5843	5855	5866	5877	5888	5899	1	2	3	5	6	7	8	9	10
39	5911	5922	5933	5944	5955	5966	5977	5988	5999	6010	1	2	3	4	5	7	8	9	10
40	6021	6031	6042	6053	6064	6075	6085	6096	6107	6117	1	2	3	4	5	6	8	9	10
41	6128	6138	6149	6160	6170	6180	6191	6201	6212	6222	1	2	3	4	5	6	7	8	9
42	6232	6243	6253	6263	6274	6284	6294	6304	6314	6325	1	2	3	4	5	6	7	8	9
43	6335	6345	6355	6365	6375	6385	6395	6405	6415	6425	1	2	3	4	5	6	7	8	9
44	6435	6444	6454	6464	6474	6484	6493	6503	6513	6522	1	2	3	4	5	6	7	8	9
45	6532	6542	6551	6561	6571	6580	6590	6599	6609	6618	1	2	3	4	5	6	7	8	9
46	6628	6637	6646	6656	6665	6675	6684	6693	6702	6712	1	2	3	4	5	6	7	7	8
47	6721	6730	6739	6749	6758	6767	6776	6785	6794	6803	1	2	3	4	5	5	6	7	8
48	6812	6821	6830	6839	6848	6857	6866	6875	6884	6893	1	2	3	4	4	5	6	7	8
49	6902	6911	6920	6928	6937	6946	6955	6964	6972	6981	1	2	3	4	4	5	6	7	8
50	6990	6998	7007	7016	7024	7033	7042	7050	7059	7067	1	2	3	3	4	5	6	7	8
51	7076	7084	7093	7101	7110	7118	7126	7135	7143	7152	1	2	3	3	4	5	6	7	8
52	7160	7168	7177	7185	7193	7202	7210	7218	7226	7235	1	2	2	3	4	5	6	7	7
53	7243	7251	7259	7267	7275	7284	7292	7300	7308	7316	1	2	2	3	4	5	6	6	7
54	7324	7332	7340	7348	7356	7364	7372	7380	7388	7396	1	2	2	3	4	5	6	6	7

LOGARITHMS OF NUMBERS (*Continued*)

Natural numbers	0	1	2	3	4	5	6	7	8	9	Proportional parts								
											1	2	3	4	5	6	7	8	9
55	7404	7412	7419	7427	7435	7443	7451	7459	7466	7474	1	2	2	3	4	5	5	6	7
56	7482	7490	7497	7505	7513	7520	7528	7536	7543	7551	1	2	2	3	4	5	5	6	7
57	7559	7566	7574	7582	7589	7597	7604	7612	7619	7627	1	2	2	3	4	5	5	6	7
58	7634	7642	7649	7657	7664	7672	7679	7686	7694	7701	1	1	2	3	4	4	5	6	7
59	7709	7716	7723	7731	7738	7745	7752	7760	7767	7774	1	1	2	3	4	4	5	6	7
60	7782	7789	7796	7803	7810	7818	7825	7832	7839	7846	1	1	2	3	4	4	5	6	6
61	7853	7860	7868	7875	7882	7889	7896	7903	7910	7917	1	1	2	3	4	4	5	6	6
62	7924	7931	7938	7945	7952	7959	7966	7973	7980	7987	1	1	2	3	3	4	5	6	6
63	7993	8000	8007	8014	8021	8028	8035	8041	8048	8055	1	1	2	3	3	4	5	5	6
64	8062	8069	8075	8082	8089	8096	8102	8109	8116	8122	1	1	2	3	3	4	5	5	6
65	8129	8136	8142	8149	8156	8162	8169	8176	8182	8189	1	1	2	3	3	4	5	5	6
66	8195	8202	8209	8215	8222	8228	8235	8241	8248	8254	1	1	2	3	3	4	5	5	6
67	8261	8267	8274	8280	8287	8293	8299	8306	8312	8319	1	1	2	3	3	4	5	5	6
68	8325	8331	8338	8344	8351	8357	8363	8370	8376	8382	1	1	2	3	3	4	4	5	6
69	8388	8395	8401	8407	8414	8420	8426	8432	8439	8445	1	1	2	2	3	4	4	5	6
70	8451	8457	8463	8470	8476	8482	8488	8494	8500	8506	1	1	2	2	3	4	4	5	6
71	8513	8519	8525	8531	8537	8543	8549	8555	8561	8567	1	1	2	2	3	4	4	5	5
72	8573	8579	8585	8591	8597	8603	8609	8615	8621	8627	1	1	2	2	3	4	4	5	5
73	8633	8639	8645	8651	8657	8663	8669	8675	8681	8686	1	1	2	2	3	4	4	5	5
74	8692	8698	8704	8710	8716	8722	8727	8733	8739	8745	1	1	2	2	3	4	4	5	5
75	8751	8756	8762	8768	8774	8779	8785	8791	8797	8802	1	1	2	2	3	3	4	5	5
76	8808	8814	8820	8825	8831	8837	8842	8848	8854	8859	1	1	2	2	3	3	4	5	5
77	8865	8871	8876	8882	8887	8893	8899	8904	8910	8915	1	1	2	2	3	3	4	4	5
78	8921	8927	8932	8938	8943	8949	8954	8960	8965	8971	1	1	2	2	3	3	4	4	5
79	8976	8982	8987	8993	8998	9004	9009	9015	9020	9025	1	1	2	2	3	3	4	4	5
80	9031	9036	9042	9047	9053	9058	9063	9069	9074	9079	1	1	2	2	3	3	4	4	5
81	9085	9090	9096	9101	9106	9112	9117	9122	9128	9133	1	1	2	2	3	3	4	4	5
82	9138	9143	9149	9154	9159	9165	9170	9175	9180	9186	1	1	2	2	3	3	4	4	5
83	9191	9196	9201	9206	9212	9217	9222	9227	9232	9238	1	1	2	2	3	3	4	4	5
84	9243	9248	9253	9258	9263	9269	9274	9279	9284	9289	1	1	2	2	3	3	4	4	5
85	9294	9299	9304	9309	9315	9320	9325	9330	9335	9340	1	1	2	2	3	3	4	4	5
86	9345	9350	9355	9360	9365	9370	9375	9380	9385	9390	1	1	2	2	3	3	4	4	5
87	9395	9400	9405	9410	9415	9420	9425	9430	9435	9440	0	1	1	2	2	3	3	4	4
88	9445	9450	9455	9460	9465	9469	9474	9479	9484	9489	0	1	1	2	2	3	3	4	4
89	9494	9499	9504	9509	9513	9518	9523	9528	9533	9538	0	1	1	2	2	3	3	4	4
90	9542	9547	9552	9557	9562	9566	9571	9576	9581	9586	0	1	1	2	2	3	3	4	4
91	9590	9595	9600	9605	9609	9614	9619	9624	9628	9633	0	1	1	2	2	3	3	4	4
92	9638	9643	9647	9652	9657	9661	9666	9671	9675	9680	0	1	1	2	2	3	3	4	4
93	9685	9689	9694	9699	9703	9708	9713	9717	9722	9727	0	1	1	2	2	3	3	4	4
94	9731	9736	9741	9745	9750	9754	9759	9763	9768	9773	0	1	1	2	2	3	3	4	4
95	9777	9782	9786	9791	9795	9800	9805	9809	9814	9818	0	1	1	2	2	3	3	4	4
96	9823	9827	9832	9836	9841	9845	9850	9854	9859	9863	0	1	1	2	2	3	3	4	4
97	9868	9872	9877	9881	9886	9890	9894	9899	9903	9908	0	1	1	2	2	3	3	4	4
98	9912	9917	9921	9926	9930	9934	9939	9943	9948	9952	0	1	1	2	2	3	3	4	4
99	9956	9961	9965	9969	9974	9978	9983	9987	9991	9996	0	1	1	2	2	3	3	3	4

ATOMIC WEIGHTS, 1926

		O = 16
Aluminum	Al	26.97
Antimony	Sb	121.77
Argon	A	39.91
Arsenic	As	74.96
Barium	Ba	137.37
Beryllium	Be	9.02
Bismuth	Bi	209.00
Boron	B	10.82
Bromine	Br	79.916
Cadmium	Cd	112.41
Calcium	Ca	40.07
Carbon	C	12.000
Cerium	Ce	140.25
Cesium	Cs	132.81
Chlorine	Cl	35.457
Chromium	Cr	52.01
Cobalt	Co	58.94
Columbium	Cb	93.1
Copper	Cu	63.57
Dysprosium	Dy	162.52
Erbium	Er	167.7
Europium	Eu	152.0
Fluorine	F	19.00
Gadolinium	Gd	157.26
Gallium	Ga	69.72
Germanium	Ge	72.60
Gold	Au	197.2
Hafnium	Hf	178.6
Helium	He	4.00
Holmium	Ho	163.47
Hydrogen	H	1.008
Illinium[1]	Il	
Indium	In	114.8
Iodine	I	126.932
Iridium	Ir	193.1
Iron	Fe	55.84
Krypton	Kr	82.9
Lanthanum	La	138.90
Lead	Pb	207.20
Lithium	Li	6.94
Lutecium	Lu	175.0
Magnesium	Mg	24.32
Manganese	Mn	54.93
Mercury	Hg	200.61
Molybdenum	Mo	96.0
Neodymium	Nd	144.27
Neon	Ne	20.2
Nickel	Ni	58.69
Nitrogen	N	14.008
Osmium	Os	190.8
Oxygen	O	16.00
Palladium	Pd	106.7
Phosphorus	P	31.027
Platinum	Pt	195.23
Potassium	K	39.096
Praseodymium	Pr	140.92
Radium	Ra	225.95
Radon	Rn	222.0
Rhodium	Rh	102.91
Rubidium	Rb	85.44
Ruthenium	Ru	101.7
Samarium	Sm	150.43
Scandium	Sc	45.10
Selenium	Se	79.2
Silicon	Si	28.06
Silver	Ag	107.88
Sodium	Na	22.997
Strontium	Sr	87.63
Sulphur	S	32.064
Tantalum	Ta	181.5
Tellurium	Te	127.5
Terbium	Tb	159.2
Thallium	Tl	204.39
Thorium	Th	232.15
Thulium	Tm	169.4
Tin	Sn	118.7
Titanium	Ti	48.1
Tungsten	W	184.0
Uranium	U	238.17
Vanadium	V	50.96
Xenon	Xe	130.2
Ytterbium	Yb	173.6
Yttrium	Y	88.9
Zinc	Zn	65.38
Zirconium	Zr	91.0

[1] The atomic weight of Illinium has not yet been reported (September 1926). It lies between the atomic weights of Neodymium and Samarium (145-146?)

INDEX

Main references are in heavy-faced type.

Abderhalden test for pregnancy, principle of, 44
Absorption, **283**
  experiments on, 287
  from gastrointestinal tract, determination of, 287
  of calcium and phosphorus from different portions of the gastrointestinal tract, influence of cod liver oil, 289
  of carbohydrate as influenced by fat ingestion, 866
  of glucose from the human stomach, study of, 287
  of sugars from the gastrointestinal tract, 287
Acacia solution, formation of emulsion by, 247
Accessory food substances, 803
  influence on growth, 803, 804, 811
Acetoacetic acid, 357, 430, 637, **656, 759,** 762
  in blood, 357
  quantitative determination of, 759, 762
  tests on, 659
Acetone, 357, 429–431, 637, **656,** 758
  bodies, 357, 429–431, 656–662, **758–763**
  determination of in urine, 758–763
  Schaffer and Marriott method for, 762
  formula for, 656
  tests for, 657–658
Acholic stool, 310
Achroo-dextrins, **106**
  α-achroo-dextrin, 106
  β-achroo-dextrin, 106
  γ-achroo-dextrin, 106
Acid, acetic, 622
  acetoacetic, 357, 637, **656,** 759, 762
  amino-acetic (see Glycocoll)
  α-amino-δ-guanidine valeric, 119, **146**
  α-amino-β-hydroxy-propionic, 120, **132**
  α-amino-β-imidazolyl-propionic, 119, **151**
  α-amino-β-indole propionic, 119, **153**
  α-amino-iso-butyl-acetic, 120, **133**
  α-amino-iso-valeric (see Valine)
  α-amino-β-methyl-β-ethyl-propionic, 119, **134**
  α-amino-normal glutaric, 120, **136**
  α-amino-β-para-hydroxy-phenyl-propionic, 120, **148**
  α-amino-β-phenyl propionic, 120, **147**
  α-amino-propionic, 119, **130**
  amino-succinic, 120, **135**
  aspartic, 120, **135**
  benzoic, 128, 612, 614, **619,** 859
  butyric, 49, 246, **622**
  cholic, 275
  chondroitin-sulphuric, 554
  combined hydrochloric (protein salt), 107, 202, 243, 884
  cyanuric, 597
  α-ε-diamino-caproic, 119, **145**
  diazo-benzene-sulphonic, 674
Acid, di-β-thio-α-propionic, 120, **142,** 685, **839**
  ethereal sulphuric, **609,** 771, 774
  fatty, 245, 246, 252, 393, **622**
  formic, 75, **622**
  free hydrochloric, 107, 202, 224, 231, 242, 243
  glucothionic, 690
  glutamic, 118, 120, **136,** 139
  glycerophosphoric, 575, 593, 623
  glycocholic, 275
  glycosuric, 619
  glycuronic, 86, 300, **662,** 744
  glyoxylic, 163
  hippuric, 128, **612,** 614, 619, 859
  homogentisic, 618
  hydrochloric, N/10 (Reagents and Solutions)
  β-hydroxybutyric, 357, 637, **656–662,** 758–763
  hydroxy-α-pyrrolidine-carboxylic, 118, 119, **141**
  kynurenic, 619
  lactic, 89, 203, 225, 238, 357, 531, **561–563,** 743
  mucic, 85, 89, 665, 666
  nucleic, 159, 188, **189,** 195–198
  organic, determination of, 706
  oxalic, **615,** 769
  oxalic, N/10 (Reagents and Solutions)
  palmitic, 245, 246, 252
  paralactic, 333, **561,** 593, **623**
  phosphoric, 631
  α-pyrrolidine-carboxylic, 115, 118, 119, **140** 176, 204, 256
  sarcolactic, 561
  stearic, 246, 576
  tannic, 93, 97, 168, 650
  taurocholic, 275
  uric, 192, 193, 197, 198, 333, 334, **377, 600, 736**
Acid albuminate (see Acid metaprotein)
Acid-base equilibrium of blood, disturbances of (table), 429
  metabolism, experiments on, 872
Acid-forming foods, influence of on hydrogen ion concentration of urine, 875
Acid-hematin, 339, 355
Acid infraprotein (see Acid metaprotein)
Acid metaprotein, 160, **180**
  coagulation of, 180
  experiments on, 180
  precipitation of, 181
  preparation of, 180
  solubility of, 180
  sulphur content of, 181
Acidities, highest total for various articles of diet, 208
Acidity of gastric juice, quantitative determination of, 217, **229,** 242
  urine, cause of, 584

Acidity of urine, cause of, quantitative determination of, by hydrogen ion concentration, 707–710
by titration, 705
Acidosis, **429,** 872
blood in, 429
general discussion of, 429–431, 872
metabolism in, 872
Acrolein, formation of, from olive oil, 250
from glycerol, 253
Acromegaly, 486, 503
Activation, 47, 257
Activation of calcium salts, 257
Adam's paper coil method for determination of fat in milk, 545
Adaptation, 107
Adenase, **45,** 192
Adenine, 45, 190, 191, **192,** 197, 626
Adequate vs optimal nutrition, 848
Addison's disease, 474
basal metabolism in, 501
Adipocere, 248
Adipose tissue, 245
Adrenal glands, 474
Adrenaline, 475, 476, 477
Comessatti reaction for, 477
crystalline form of, 475
determination of, 476
experiments on, 476
formula for, 474
preparation of, 476
Vulpian reaction for, 476
Adsorption, 4, 50, 167
by cellulose and charcoal, 17
Agar-agar, 67, **100,** 311, 792
Agglutination, 336, 347
Air, analysis of, 529
alveolar, 499
carbon dioxide in expired, 431
complemental, 499
composition of, 498, 499
evaporation in, 493
expired, 499
inspired, 499
residual, 499
supplemental, 499
tidal, 499
Alanine, 119, 130
Albino rats, activity cage for, 795
cages for care for, 793–796
care and caging of, 792
data sheet for, 797
experiments on, 828–849
food chart for, 797
dish for, 794
growth chart for, 798
metabolism cage for, 795, 796
Albumin, egg, 158, 162, 170, 172
crystalline form of, 171
crystallized, preparation of, 170
molecular weight of, 114
powdered, preparation of, 172
tests on, 172
serum, 158, 162, 331, 333, 354
solution, preparation of, 162
Albumin in urine, 644, 754–758
determination of, 754–758
determination of, official method, 756
standards, preparations of permanent, 756
Albumin in urine, tests for, 644
Albuminates (See Metaproteins)
Albuminates, formation of, by metallic salts, 166
Albuminoids, 158, **177**
Albuminometer, Esbach's, 755
Albumins, 158, 160, 162, 170–172
Albumoscope, 168, 646
Albumoses (see Proteoses)
Alcaptonuria, 619
Alcohol, aldehyde test for, 79
iodoform test for, 79
Alcohol-soluble proteins (see Prolamins)
Aldehyde, 74, 90
Aldehyde group, 90
Aldehyde test for alcohol, 79
v. Aldor's method of detecting proteose in urine, 649
Aldose, 66
Aliphatic nucleus, 115, 119
Alizarin, 12, *222–224*
Alizarin red, 12, 222
Alizarin yellow R, use of, as indicator, 12, 222
Alkali albuminate (see Alkali metaprotein)
Alkali-hematin, 339
Alkali metaprotein, 160, **180,** 181
experiments on, 181
precipitation of, 181
preparation of, 181
sulphur content of, 181
"Alkali reserve," determination of, 432, 441, 443, 446
Alkali tolerance; determination of, 447
Alkaline tide, **875**
demonstration of, 875
Alkalosis, 448
Allantoin, 616
experiments on, 617
formula for, 616
quantitative determination of, by Wiechowski-Handovsky method, 740
by difference, 742
Almén's reagent, preparation of, 651
Alumina cream, use of, 167
Aluminum hydroxide, use of, in removal of protein, 548, 713, 882
Alveolar air, carbon dioxide tension of, 431
determination of, 441
Amalgamation test for mercury, 670
Amandin, 158
Amino acid deficiency, influence of, 838
Amino acids, 113, 118, 119, 120, **121,** 333, 380, 618, 728
crystalline forms of, 130–154
essential, 126
experiments on, 129–154
in blood, determination of, 380
preparation of, in crystalline form, 130, 134, 139, 143, 144, 149
unessential, 126
Amino group, 115, 117, 122, 164
α-amino-δ-guanidine valeric acid (see Arginine)
α-amino-β-hydroxy-propionic acid, 120, **132**
α-amino-β-imidazol-propionic acid, 119, **151**
α-amino-β-indole propionic acid, 119, **153**
α-amino-iso-butyl-acetic acid, 120, **133**
α-amino-iso-valeric acid (see Valine)
Amino-nitrogen, quantitative determination of, 380, 728
α-amino-normal-glutaric acid, 120, **136**

Amino-succinic acid, 120, **135**
Ammonia, 118, 172, 333, 334, **627, 724**
in blood, 333, 334
in proteins, 118, 176
in urine, **627, 724**
quantitative determination of, 724
Ammoniacal cupric hydroxide, solubility test, 99
Ammoniacal silver solution, preparation of, 882
Ammonium benzoate, synthesis of, to form hippuric acid, 859
Ammonium magnesium phosphate ("Triple phosphate"), 571, 633
crystalline form of, 633
in urinary sediments, 681
Ammonium purpurate, 193, 604
Ammonium thiocyanate, standard solution, preparation of, 882
Ammonium urate, 585, 601, 684
crystalline form of, Plate VII, opposite p. 685
Amphopeptone, 160, 184
Amygdalin, 45
Amylase, pancreatic, 45, 56, 258, 262
digestion of dry starch by, 258, 262
inulin by, 262
experiments on, 56, 262
influence of bile upon action of, 262
most favorable temperature for action of, 262
salivary, 45, 55, **106,** 202
activity of, in stomach, 107, 202
experiments on, 55, 108
inhibition of activity of, 107, 111
nature of action of, 106
products of action of, 106, 110
tests for kidney efficiency, 678
vegetable 45, 56
Amylases, 45, 55, 106, 258, 262
experiments on, 55, 110, 262, 325
preparation of (Sherman), 56
Amylocellulose, 92
Amyloid, 98, 178
Amylolytic enzymes (see Amylases)
quantitative determination of activity of, 263, 325, 678
Amylopectin, 92
Amylose, 92
Animal calorimetry, physical and chemical bases of, 492
parasites in feces, 315
in urinary sediments, 697
Anti-enzymes, 50
experiments on, 64
Antiketogenesis, 431, **873**
Antiketogenic, 431, **873**
Antimony pentachloride as cellulose solvent, 99
Antimony trichloride as cellulose solvent, 99
Antipepsin, 50, 64
Antipeptone, 160, 184
Antirachitic factor (see Vitamin D)
activation of foodstuffs by ultraviolet light, 830
vitamin (see Vitamin D)
Antirennin, 50
Antiscorbutic vitamin (see Vitamin C)
Antithrombin, 342
Antitrypsin, 50, 64
Arabinose, 67, **86**
Bial's reaction for, 86, 664
Arabinose, phenylhydrazine test on, 86
Tollens' reaction on, 86, 664
Arginase, 45
Arginine, 45, 118, 119, **146,** 256
Aromatic oxyacids, 618
Arsenic in urine, 668
Arterial blood, composition of, 499
Asbestos, for suction filters, 882
Ascaris, 64
Ash of milk, quantitative determination of, 546
Askenstedt, method for indican, Parker's modification of, 765
Asparagine, 136
Aspartic acid, 118, 119, **135**
crystalline form of, 136
formula for, 135
Atmospheric air, composition of, 498
Atomic weights, table of, 905
Atwater-Rosa-Benedict respiration calorimeter, 510
Aub and DuBois, standards of, 504
Autocoids, 474
Autolytic enzymes, 45
Automatic regulation of gastric acidity, 216, 220
Autoxidizable substance, 53
Avitaminosis, 803
Avogadro, law of, 493

Babcock fat method, 544
tube, 544
Bacteria in feces, 308, 313, 327, 861
quantitative determination of, 327
Bacterial nitrogen in feces, 314, 861
determination of, 327
Baeyer hypothesis, 68
Bailey gasometer, 520
gas sampling bottles, operation of, 522
modification of gas analysis apparatus, 523, **524**
Balance, metabolic, preparation of, 870
calcium, 870
magnesium, 870
nitrogen, 870
phosphorus, 870
sulphur, 870
Barcroft manometer, 51
Barfoed's reagent, preparation of, 77, 271, 883
Barfoed's test for monosaccharides, 77
Baryta mixture, preparation of, 883
Basal metabolic rate, 509
definition of, 509
Basal metabolism, 500
calculations of, 504
clinical interpretation of, 500
definition of, 509
determination of by gasometer method, 518
standards of, 504
Aub and DuBois, 504
Harris-Benedict, 507
Base-forming foods, influence of on hydrogen ion concentration of urine, 875
Basic lead acetate solution, 883
Bauer's test to differentiate human and cow's milk, modification of, 539
Bayberry tallow, saponification of, 251
source of, 251
Bayberry wax (see Bayberry tallow)
Bead test (Einhorn), 324
Beckmann-Heidenhain apparatus, 588

Beeler (*et al.*) carbohydrate tolerance test, 391
Beer's law, 359
Bell and Doisy method for phosphates in blood, 403
in urine, 776
Bence-Jones protein, crystalline form of, 648
detection of, 650
molecular weight of, 114
quantitative determination of, 758
Benedict and Bock's method for quantitative determination of total nitrogen, 716
Benedict-Collin's respiration calorimeter, 513
Benedict cot chamber, 511
Benedict creatine preparation, 609
Benedict-Folin creatinine preparation, 607
Benedict-Folin method for creatine in urine, 734
Benedict and Fox's oxycalorimeter, 853
Benedict and Franke's method for uric acid in urine, 736
Benedict modification of the Folin and Wu blood sugar method, 383
Benedict portable respiration calorimeter, 512
Benedict's method for quantitative determination of sugar, 744
Benedict's method for quantitative determination of sulphur, 772
Benedict's method for the quantitative determination of uric acid in blood, 377
Benedict's method for sugar in normal urine, 754
Benedict and Osterberg's method for sugar in normal urine, 753
Benedict's picric acid method for sugar in blood, 387
Benedict's reagent, for use in quantitative determination of sugar, preparation of, 883
Benedict-Roth respiration apparatus, 512, **513**
Benedict's solution, preparation of, 883
student respiration apparatus, **515**, 516
sulphur reagent, preparation of, 884
Benedict and Theis method for determination of inorganic phosphate in blood, 406
Benedict and Theis method for determination of lipoid phosphorus in blood, 395
Benedict and Theis method for determination of phenols in blood, 400
Benzidine peroxidase reaction (Wilkinson and Peters), 540
Benzidine reaction for blood, 239, 320, 348, 651
Benzoic acid, 128, 612, 614, **619**, 859
experiments on, 620
Bergeim's intragastric conductance apparatus, 219
Bergeim's modification of the Herter-Foster method for indole in feces, 328
Beri-beri, 803, 811
Berthelot-Atwater bomb calorimeter, 852
Bial's reaction for pentoses, 86, 664
Bial's reagent, preparation of, 86, **664**, 884
Bicarbonate of plasma, titration, 437
Bicolorimeter, Myers, 26, 362
Bicolorimetric methods for hydrogen ion concentration of the urine, 709
Bile, 273, 654
analysis of, 274
cholesterol in, 274, 278, 281
constituents of, 274
daily secretion of, 273
freezing-point of, 274
influence on digestion, gastric, 213
pancreatic, 261
Bile, inorganic constituents of, 274, 278
nucleoprotein of, 274, 278
reaction of, 273, 278
secretion of, 273
specific gravity of, 274
Bile acids, 275, 279, 280, 323, 654
tests for, 279
Bile acids in feces, detection of, 323
Bile acids in urine, 655
tests for, 655
Bile pigment in serum, determination of, 396
Bile pigments, 274, 276, 278
Kapsinow's test for, 655
tests for, 278
Bile pigments in urine, 654
tests for, 654
Bile salts, 274
crystallization of, 276, 280
Biliary calculi, 278, 280
analysis of, 280
Bilirubin, 276
crystalline form of, 276
in urinary sediments, 687
Biliverdin, 276, 277
"Biological" blood test, 342
Bios, 815
"Bios 223," 815
Bismuth reduction test, 77, 642
Bismuth test for choline, 580
Biuret, **164, 597**
formation of, from urea, 164. **597**
Biuret paper of Kantor and Gies, 165, 884
Biuret potassium cupric hydroxide (see Cupri-potassium biuret), 165
Posner's modification of, 166
test, 164
Biuret reagent (Gies), preparation of, 165, 884
Black tongue, 816
Black's reaction for β-hydroxybutyric acid, 661
reagent, preparation of, 661
Blood, 239, 320, **331, 356**, 651
acid-base equilibrium of, disturbances of, 429
analysis, 356
arterial, composition of, 499
calcium in, 357, 408
carbon monoxide in, determination of, 457
manometric determination of, 468
chemistry of, 356
coagulation of, 341
Howell's theory of, 342
composition of normal and pathological, 357
constituents of normal and pathological, 333, 334, **357**
crystallization of oxyhemoglobin of, 336, 351
defibrinated, 342, 345
detection of, 239, 320, 342, **348**, 651
in feces, 320
in gastric contents, 239
in urine, 651
drawing of, for analysis, 366
experiments on, 345, 356
finger-tip, determination of glucose in, 385
for analysis, drawing of, 366
in disease, 357 (table)
"laked," 331, 346
manometric determination of carbon dioxide in, 464
of urea in, 470

Blood, methemoglobin in, manometric determination of, 469
menstrual, 342
microscopical examination of, 345
oxygen in, manometric determination of, 466
plasma, 331, 333, 354
Cullen's method for hydrogen ion concentration of, 448
phenols, determination of, 400
preservation of, for analysis, 366
protein, determination of, 398
reaction of, 331, 345
serum, 333, 353
spectroscopic examination of, 414
stains, 354
total sulphate in, determination of by method of Denis and Leche, 407
urea, determination of by manometric gas analysis, 470
venous, composition of, 499
Blood analysis, 356
Blood casts in urine, 692
Blood in urine, 651
tests for, 651
Blood plasma, 331, 333, 354
constituents of, 333
effect of calcium on oxalated, 354
experiments on, 354
preparation of fibrinogen from, 354
oxalated, 354
salted, 354
Blood serum, 333, 353
experiments on, 353
Blood stains, examination of, 354
Bloor, Pelkan and Allen's method for determining fatty acids and cholesterol in blood, 393
Bloor's method for determination of lipoid phosphorus in blood, 395
nephelometer, cut of, 364
Bock and Benedict's apparatus, cut of, 716
Bock and Benedict's colorimeter, 361
Bock and Benedict's microchemical method for total nitrogen in urine, 716
"Bolting" of food, influence of, on food residues in feces, 862
Bomb calorimeter, Berthelot-Atwater, 852
Bone, constituents of, 555
ossein of, preparation of, 555
quantitative composition of, 555, 556
Bone ash, scheme for analysis of, 557
use of in diet, 801
Boneblack, purification of, 884
Boothby and Sandiford's surface area nomogram, 505
Borchardt's reaction for fructose, 84, 667
Bordet test, detection of human blood by, 342
Boric acid and borates in milk, detection of, 543
Boyle's law, 492
Brass scale correction for barometer, 894
Brownian movement, 2
Bromelin, 46
Bromine test for melanin, 672
for tryptophane, 260
Buccal glands, 104
Buffer solutions of Clark and Lubs, **13**
"Buffer substances" of the blood, 420
Buffy coat, formation of, 333
Bunge's mass action theory, 203
Burge apparatus for quantitative determination of catalase, 63
Butter, composition of, 247, **537, 804**
Butyric acid, 49, 246, **622**
Bynin, 158, 176

Cadaverine, 295
formula for, 292
Calcium balance, preparation of, 870
carbonate in urinary sediments, 682
caseinate, 537
deficiency, 846
in blood, 357
in blood determination of, 408
in bone, detection of, 556, 557
in feces, estimation of, 869
oxalate, 615, 681
in urinary sediments, 681
paracasein, 205, 533
paracaseinate, 533
phosphate in urinary sediments, 683
in bone, detection of, 556, 557
in milk, 530, 541
in serum, Clark-Collip method for determination of, 408
sulphate in urinary sediments, 683
in tissues, method of Corley and Denis for determination of, 409
in urine, 631, 634, 784
determination of, 784
Shohl and Pedley's method for determination of, 784
Calcium and phosphorus, absorption of, 289
utilization of, 288
Calcium-depositing substances, line test for, 838
Calcium-pigment calculi in bile, 278
Calculi, biliary, 278, 280
urinary, 698
Calomel electrodes, standard values for, 32
Calorimeter, Atwater-Rosa-Benedict, 510
Benedict-Collins, 513
Benedict and Fox oxy-, 853
Benedict portable, 512
Benedict-Roth, 513
Benedict student, 515
heat of combustion in, 494
universal, 511
Calorimetry, animal, physical, and chemical bases of, 492
animal, 492, **510**
Camphor as urine preservative, 591
Cane sugar (see Sucrose)
Carbohydrases, 45
Carbohydrate deficiency, influence of, 842
demonstration on, 842
metabolism, experiments on, 863
tolerance tests, 388, 391
utilization, influence of diet on, 865
utilization, influence of fasting on, 864
Carbohydrate tolerance tests, 388, 391
Carbohydrates, 66, 621
absorption of, as influenced by fat ingestion, 866
classification of, 67
composition of, 66
in feces, estimation of, 866
in urine, 621
oxidation of, 494
protein-sparing action of, 851

Carbohydrates, review of, 101
scheme for detection of, 103
specific rotation of, 81
transformation of, into fats and proteins, 250
variation in solubility of, 66
Carbon dioxide, of expired air, demonstration, 431
capacity of plasma, 432, 465
tension of alveolar air, 439, 441
manometric determination of in blood or plasma, 464
Carbon moiety of protein molecule, 248
Carbon monoxide hemoglobin, 339
in blood, manometric determination of, 468
determination of, 416, 457, 468
spectroscopic test for, 415
tannin test for, 416
Carbonates in urine, 635
Carboxylase, 45, 51, 79, 643
Carmine, use in feces separation, 325, 791
Carmine-fibrin, preparation of, 57
Carotin, 333, 538
Cartilage, 554
experiments on, 554
Casein, 19, 46, 159, 178, 205, **530–537**
action of rennin upon, 205, 533
decomposition of, 118, 119, 120
experiments on, 541
isoelectric point and solubility of, 19
quantitative determination of, 547
Caseinate, calcium, 537
Caseinogen (See Casein.)
Castration, influence of, 486
Casts in urinary sediments, 690–695
Catalase, 45, 63
experiments on, 63
quantitative determination of, 63
Catalysis, 42
Catalysts, inorganic, experiments on, 55
Cataphoresis, 6
Catherization, collection of urine of dogs by, 790
Cellulose, 67, 97
adsorption of dyes by, 17
experiments on, 98
structural formula of, 97
Cellulose group, 67
Cerebrin (cerebroside), 575, 577, 579
experiments on, 579
Cerebrosides, 575, 577
Cerebro-spinal fluid, choline in, 576
Chalones, 474
Charcoal, adsorption by, 17
Charles, law of, 493
Charcot-Leyden crystals, 312
form of, 312
Chloride shift, 423
Chlorides in blood, **357**, 401
quantitative determination of, 401, 402
in urine, 630, 780
detection of, 631
quantitative determination of, 780
Chlorophyll, 68
as photocatalyst, 68
Cholesterol, 274, 278, 281, **357**, 391, **577**, 686
crystalline form of, 281
formula for, 577
in blood, 357
determination of, 391
Cholesterol, isolation of, from biliary calculi, 280
occurrence of, in urinary sediments, 686
origin of, 578
preparation of, from nervous tissue, 579
tests for, 281, 579
Cholesterol-calcium calculi, 278
Cholesterol-calcium-pigment calculi, 278
Choletelin, 276
Cholic acid, 275
Choline, **576**, 577, 580
formula for, 576
tests for, 580
Chondrigen, 178
Chondroalbumoid, 554
Chondroitin, 554
Chondroitin-sulphuric acid, 554
Chondromucoid, 178, 554
Chondrosin, 554
reducing action of, 554
Chromoproteins (see Hemoglobins)
Chyle, 345
Clark and Lubs, buffer solutions of, 11, **13**
Clark-Collip, autoclave method for urea in blood, 372
method for determination of calcium in serum, 408
Clausen method for determination of lactic acid in blood, 400
method for determination of lactic acid in urine, 743
Cleaning solution, 884
Cleavage products of protein (see Decomposition products)
Clupeine, 118, 159
Coagulases, 45
Coagulated proteins, 115, 160, **181**
biuret test on, 183
digestion of, 183
formation of, 116, 182
Hopkins-Cole reaction on, 183
Millon's reaction on, 183
solubility of, 183
xanthoproteic reaction on, 183
Coagulation of blood, 341
Howell's theory of, 342
Coagulation of proteins, 181
changes in composition during, 182
fractional, 169, 181
Coagulation temperature of proteins, 169, 181
apparatus used in determining, 170
method employed in determining, 169
Cochineal solution, preparation of, 884
indicator for total phosphates in urine, 778
Cod liver oil, 247, 248, 289, 804, 806, 808, 810, 824, 829, 835, 837, 841
antirachitic factor of, 248, 804, 824, 835
relationship of ultraviolet light to, 808, 829, 830
as a source of vitamins, 804, 806, 807
as a tonic, 823
color reactions for, 810, 811, 824
"concentrates," 808, 837
influence of, on absorption of calcium and phosphorus, 289
irradiation of, 808
potency units of, 809
preservation of vitamin of, 808

Cod liver oil, prevention and cure of rickets by, 823, 828, 837
rickets cured by, 823, 828, 837
ultraviolet light and, 808, 829, 830
unsaponifiable portion of, 807
use of, in diets, 806, 807, 823, 836, 841
U. S. P. assay for, 808
care and caging of animals used in, 792
vitamin A in, 247, 248, 804, 808, 810, 811, 836
color reactions for, 810, 811
preservation of, 808
vitamin D in, 248, 804, 824, 835
color reactions for, 824
vitamins in, 804
Co-enzyme, 47
Collagen, 158, 177, 550, 551
experiments on, 552
percentage of, in ligament, 553
in tendon, 550
production of gelatin from, 550, 552
solubility of, 552
Collection and preservation of feces in metabolism experiments, 790
of urine in metabolism experiments, 590, 789
Collodion dialyzer, 73
Colloid, protective, demonstration of, 16
Colloidal, ferric hydroxide, preparation of, 15
gel, 1
iron, use of, 167
particles, 1
platinum, preparation of, 15
solutions, 1, 7, 531
experiments on, 15
methods for preparation of, 15
osmotic pressure of, demonstration of, 18
viscosity (internal friction) of, 17
Colloids, 531, 560
mutual precipitation of, 16
optical properties of, 16
precipitation of, by salts, 16
tissue, 560
Colon, absorptive power of, 286
Colorimeters, 358–363
Bock-Benedict, 361
Duboscq-Spencer, 361
Hellige-Leitz, 360
Myers, 360
Colorimetry, 358
calculations, 359
Colostrum, 532, 535, **538**
microscopical appearance of, 532
Combination, 298
Combined hydrochloric acid (protein salt), 107 202, 884
preparation of, 884
tests for, 243
Combustion of foodstuffs, 853
Comessatti reaction for adrenaline, 477
Comparator block, 24, 361
Composition of common foods (Table), 850
Conductance of solutions, determination of electrical, 38
Conductance, specific, 40
Congealing-point of fat, 254
Congo red, as indicator, **12**, 223
preparation of, 884
Congo-red fibrin, preparation of, 57, 884
Conjugate glycuronates, 300, 662
naphthoresorcinol reaction for, 662
polariscopic-fermentation test for, 662
reduction-polariscopic test for, 663
Conjugated proteins, 159, **177**
classes of, 159, **177**
nomenclature of, 159, **177**
occurrence of, 159, **177**
Conjugation, 298
Connective tissue, 549, **550**
experiments on, 551–557
Copper soap test for lipase, 264
Corley and Denis method for determination of calcium in tissues, 409
Cowgill's procedure for metabolism experiments on dogs, 799
salt mixture, 801
Cowie's guaiac test, 322
Creatine, 333, 357, 360, **564**, 568, **572**, 607, **609**, 734
crystalline form of, 564
diacetyl reaction for, 572
formula for, 568
preparation from creatinine, 609
quantitative determination of, 734
separation of, from meat extract, 572
tests on, 572, 607
transformation into creatinine, 564
Creatinine, **357**, 375, **605**, 732
daily excretion of, 605
determination of, in blood, 375
in urine, 732
elimination, a study of, 859
experiments on, 607
Jaffe reaction for, chemistry of, 609
separation of, from urine, 607
Creatinine-zinc chloride, formation of, 608
Cretin, the effect of thyroxin on growth of, 480
Cretinism, 480
Cross and Bevan's reagent, 99
preparation of, 99
solubility test, 99
Cryoscopy, 588
Cullen's method for hydrogen ion concentration of blood plasma, 448
Cupri-potassium biuret, formation of, 165
formula for, 165
Cyanamin, 202
Cyanosis, 452
Cyanuric acid, 597
formula for, 597
Cylindroids in urinary sediments, 695
α-Cyprinine, 118
Cysteine, 299
formula for, 142
qualitative tests for, 145
Sullivan's test for, 145
Cystine, 115, 118, 120, **142**, 685, **839**
biological assay of, 839
crystalline form of, 143, 685
deficiency, demonstration of, 839
detection of, 144
formula for, 142
in hair, 143
in urinary sediments, 685
preparation of, in crystalline form, by Folin's method, 143
by Schmidt's method, 144

Cystine, relation of to growth, 841
Sherman biological test for, 144
Cytosine, 190, 191, 193, 199
Wheeler-Johnson reaction for, 199

Deaminases, 45
Decomposition products of proteins, 113, 115 118, 119, 120
crystalline forms of, 130–154
experiments on, 129–154
isolation of, 129–154
Defensive enzymes, 43
Deficiencies of diet, 803
Deficiency diseases, 803
Degradation products of protein (see Decomposition products)
Denis and Leche method for determination of total sulphate in blood, 407
Denis method for determination of magnesium in serum, 410
for inorganic sulphate in blood, 406
Derived proteins, 159, **179**
Detection of preservatives in milk, 542
Detoxication, 296
and putrefaction, 291
putrefaction and reduction, experiments on, 303
Deuteroproteose, 160, 184
Dextrin, 67, **96**, 106
α-achroo-, 106
β-achroo-, 106
γ-achroo-, 106
erythro-, 96, 106
experiments on, 96
in presence of starch, detection of, 103
Dextrosazone, crystalline form of, Plate III, opposite p. 72
Dextrose (see Glucose)
Diabetes, renal, 383
Diacetic acid (see Acetoacetic acid)
Dialysis, 2, 16, 73, 74
Dialyzers, preparation of, 74
Diamino acid nitrogen, 116
Diastase (see Vegetable amylase)
Diazo reaction (Ehrlich's), 674
type A and type B, 674
Diazo-benzene-sulphonic acid, 674
reagent, preparation of, 674
Diet, adequate, 830, 848
vs optimal, 848
amino acid deficiency in, 838
calcium deficiency in, 846
carbohydrate deficiency in, 842
deficiencies, 803
energy deficiency in, 845
fat deficiency in, 844
protein deficiency in, 838
vitamine deficiency in, 803, 830, 831
water deficiency in, 847
Dietary deficiencies and vitamins, 803
experiments on, 830
Differentiation between pepsin and pepsinogen, 203, 212
Diffusion through gels, 16
through membranes, 16
Digestion, gastric, 200
intestinal, 266
pancreatic, 255
salivary, 104
Di-methyl-amino-azobenzene (see Töpfer's reagent)
Dipeptides, 116, 160
Direct aeration method for urea in blood, Myers', 374
Direct nesslerization for nitrogen of urine, Koch and McMeekin's method, 717
of blood, Koch and McMeekin's method, 370
Disaccharides, 67, **87**
classification of, 67
Dissociation products of protein (see Decomposition products)
Dogs, Cowgill's procedure for metabolism experiments, 799
Donnan equilibrium, 4, 5
demonstration of, 19
Donné's pus test, 653
Double hydrogen electrode system, 34
quinhydrone electrode system, 36
Dreyer, standards of, 507, 510
Drop method, determination of surface tension by, 22
Drying method for determination of total solids in urine, 710
Du Bois surface area chart, 506
Du Nouy surface tension apparatus, 23
Duboscq-Spencer colorimeter, 361
Dyes, adsorption of, by cellulose, 17

Earthy phosphates in urine, **631**, 633, 778
quantitative determination of, 778
Edestan, 159, 180
Edestin, 118, 158, **174**
coagulation of, 175
decomposition of, 118
tests on crystallized, 174
Egg albumin, crystalline form of, 171
Ehrlich's diazo-benzene-sulphonic acid reagent, preparation of, 885
Ehrlich's diazo reaction, 674
Einhorn's bead test, 324
Einhorn's saccharometer, 79
Elastin, 158, 177, 552, 553
experiments on, 553
Electrical conductance of solutions, determination of, 38
conductivity of urine, 589
Electrode, calomel, 32
system, double hydrogen, 34
quinhydrone, 36
Electrolytes, influence on enzyme activity, 48, 258
Electrometric determination of hydrogen ion concentration, 27
method for the hydrogen ion concentration of urine, 710
oxidation and reduction titrations, 37
titration of acids and bases using hydrogen electrode, 32
determination of hydrogen ion concentration by, 34
method of Meeker and Oser for hydrogen ion concentration, 34, 36
Emulsin, 45
Emulsoid solutions, preparation of, 16
state, 3
Emulsoids, 3

Endocrine glands, 473
organs, 473
system, schematic chart of, 473
Energy deficiency, influence of, 845
demonstration on, 845
metabolism, 491
Enol form of uric acid, 600
Enterokinase, 266, **267**, 268
demonstration of, 268
Enzymes, **41**
destructive action of ultraviolet light on, 48
experiments on, 55
influence of electrolytes, 48, 258
ultraviolet light on, 48
list of, 45
preparation of, 46, 57, 58, 59
properties of, 47
reference books, 41
Epinephrine (see Adrenaline)
Epithelial cells in urinary sediments, 688
casts in urinary sediments, 692
Epithelial tissue, 549
experiments on, 550
Erepsin, 45, **266**, 269
experiments on, 269
Erythrocytes, 335, 346, 353, 695
crenated, 696
influence of osmotic pressure on, 346
in urinary sediments, 695
of different species, 335
Erythro-dextrin, 96, 106
Esbach's albuminometer, 755
method for determination of albumin, 755
reagent, preparation of, 885
Ethereal sulphates, **609**, 771, 774
quantitative determination of, 771, 774
Ethereal sulphuric acid, **609**, 771, 774
Ethyl butyrate test for pancreatic lipase, 264
Euglobulin, 331, 332
Evacuation time of foods, 207–209
for fast and slow stomachs, 209
Evaporation in air, 493
Excelsin, 175
crystalline form of, 175
Exercise, effect of on the composition of urine, 585, 867
Experimental animals, dogs as, 799
mice as, 802
rats as, 792
Exton's reagent, 646
test for albumin, 646
Extractives of muscular tissue, 560
nitrogenous, 560
non-nitrogenous, 560

Factor P-P, 803, 804, **815**
sources of, 804, 815
Farmer-Folin microchemical method for nitrogen in urine, 714
Fast stomach, evacuation of food by, 209
Fasting, feces in, 230
metabolism in, 880
Fat deficiency, influence of, 844
demonstration on, 844
metabolism, experiments in, 868
Fatigue substances of muscle, 371
Fats, 245
absorption of 248, 258, **285**
Fats, apparatus for determination of melting-point of, 253
chemical composition of, 245, 246
congealing-point of, 254
crystallization of, 245, 251
digestion of, 248, 258, **285**
emulsification of, 247, 251, 285
experiments on, 250
formation of, from protein, 249, 250
formation of acrolein from, 250
hydrogenation of, 246
hydrolysis of, 246
influence of, on carbohydrate absorption 865, 866
in milk, 530, **535**, 542, **544**
in urine, 664, 692
melting-point of, 247, 253
neutral, 246, 247, 250
nomenclature of, 245, 246
occurrence of, 245
oxidation of, 495
permanent emulsions of, 247, 251
protein-sparing, action of, 851
quantitative determination of, in milk, 544
rancid, 247, 250
reaction of, 247
saponification of, 246, 252, 285
solubility of, 247, 250
tide, 285
transitory emulsions of, 247, 251
utilization, 868
Fat-soluble "A," (see Vitamin A)
Fat-splitting enzymes (see Lipases)
Fatty acid, 245, 246, 252, 393, **622**
Fatty casts in urinary sediments, 692
Fatty degeneration, 248
Fearon's color tests for vitamin A, 811
Fecal amylase, quantitative determination of, 325
Fecal bacteria, 291, 308, 313, 327, 861
quantitative determination of, 327
Feces, **308**, 327, 790, 861
agar-agar, influence of, 311, **792**
bacteria in, 308, 313, 327, 861
bacterial nitrogen in, demonstration of, 327, 861
collection and preservation in metabolism experiments, 790
experiments on, 316, 851, 861, 862, 866, 868, 869
food residues in, 862
influence of defective mastication on, 862
inorganic elements of, demonstration of, 869
metabolic product nitrogen in, 862
Fehling's method for determination of glucose in urine, Benedict's modification of, 744
solution, preparation of, 885
test, 75, 639
Benedict's modification of, 76, 641
Fermentation, lactic acid, 238, 531
"sugar-free," 51, 79, 643
Fermentation method for determination of glucose, 751
Fermentation test, 79, 643
Ferments, classification of, 45
Ferric chloride test for acetoacetic acid, 659
for melanin in urine, 672
for thiocyanate in saliva, 109
Fibrin, 332, 341, 354
carmine, preparation of, 57

Fibrin, congo-red, preparation of, 57
in urinary sediments, 697
separation of, from blood, 341, **354**
solubility of, 354
Fibrin ferment, 341
Fibrin-heteroproteose, 119
Fibrinogen, 332, 341, 342
Finger nails, composition of, 549
Finger-tip blood, determination of glucose in, 385
Fiske's method for determination of total fixed base, 783
Fiske and Subarrow's method for determination of inorganic phosphate in blood, 403
of total acid-soluble phosphorus in blood, 405
phosphate, 776
Fluorides in urine, 636
Fly-maggots, experiments on, 249
Foam test for bile acids, 280, 655
Folin apparatus for nitrogen, etc., 713, 715
Folin fume absorber, 711
Folin's blood sugar method, 384
gravimetric method for determination of protein in urine, 754
method for determination of acetone in urine, 763
acidity of urine, by titration, 705
amino-acid nitrogen in blood, 380
amino-acid nitrogen in urine, 731
ammonia, 724
creatine in urine, 735
creatinine in urine, 732
ethereal sulphates, 771
inorganic sulphates, 771
total sulphates, 770
of preparing cystine, 143
Folin's sugar reagent, 622
test for sugar in normal urine, 621
test for uric acid, 604
theory of protein metabolism, 861
Folin and Bell's method for determination of ammonia in urine, 727
Folin-Benedict method for determination of creatine, 734
Folin, Benedict and Myers' method for determination of creatine, 735
Folin and Berglund's method for sugar in normal urine, 621, **753**
Folin, Cannon and Denis method for determination of adrenalin, 476
Folin and Denis' direct nesslerization method for nitrogen in urine, 718
Folin and Denis' method for Bence-Jones protein, 758
Folin and Denis' method for phenols in urine, 766
Tisdall's modification of, 768
Folin and Denis' turbidity method for albumin in urine, 756
Folin-Farmer microchemical method for total nitrogen in urine, 714
Bock and Benedict's modification of, 716
Folin and Macallum's microchemical method for Foammonia, 726
Folin-McEllroy reagent, 76, 641, 885
Folin-McEllroy test for sugar, 76, 641
Folin-McEllroy-Peck method for determination of sugar in urine, 746
phosphate-carbonate-thiocyanate reagent, 746
Folin-Osborne method for total sulphur in urine, 773
Folin-Wright method for total nitrogen in urine, 713
Folin-Wu, system of blood analysis, 366
method for determination of creatine in blood, 376
creatinine in blood, 375
glucose in blood, 381
non-protein nitrogen in blood, 368
sugar in blood, 381
urea in blood, 370
uric acid in blood, 378, 380
urine, 737
Folin and Youngburg direct nesslerization method for urea in urine, 722
Foods, acidities for various, 207–209
composition of, 850
evacuation time of, 207–209
purine content of, 857
Foreign substances in urinary sediment, 697
Form elements of blood, 331
Formaldehyde, 68
as milk preservative, 542
Formaldehyde-H_2SO_4 test (Mörner), 150
Formic acid, 75, **622**
Formol titration method of Malfatti, 727
of Sörensen, 728
Fractional coagulation of proteins, 169, 181
method of gastric analysis, 217, 224
Free hydrochloric acid, 107, 202, 231, 242, 243
test for, 224
Fridericia apparatus, 442
Fridericia's method for determination of alkali reserve, 441
Fructose, 67, **83**
Borchardt's reaction for, 84, 667
in urine, 667
phenylhydrazine test for, 668
relative sweetness of, 71
Seliwanoff's reaction for, 667
Fuchsin-frog experiment, 569
Fuel value of food, determination of, 853
Furfural, formation of, 72
solution, preparation of, 885
Fusion mixture, preparation of, 194, 885

Galactans, 67, 100, 191
Galactase, 538
Galactosamine, 178
Galactose, 67, 84, 666,
experiments on, 85
relative sweetness of, 71
Gas analysis apparatus, Bailey's modification of, 523, **524**
principles of, 523
procedure, 526
Gasometer, Bailey, 520
Gasometer method for collection of expired air, 519
for basal metabolism, 518
Gastric acidity and the use of indicators, 12, **221**
automatic regulation of, 202, 216
Gastric analysis, 217
detection of bile in, 240
of blood in, 239
of food rests in, 241
of lactic acid in, 238
of mucus in, 241

Gastric analysis, determination of free acidity in, 231
of peptic activity in, 234
of total acidity in, 229
of tryptic activity in, 237
examination of samples in, 229
fractional method of, 217, **224**
introduction of the tube in, 225
Rehfuss tube, use of in, 217, 225
retention meal in, 228
test meals in, 228
use of indicators in, 221
Gastric digestion, 200
conditions essential for, 211
general experiments on, 211
influence of bile on, 213
influence of different temperatures on, 212
influence of water on, 200, 210
most favorable acidity for, 212
power of different acids in, 212
products of, 204, 211
Gastric fistula, 201
Gastric juice, 203, 210
acidity of, 202, 217
automatic regulation of, 216
influence of water on, 200, 210
of regurgitation on, 202, 206, 216, 220
analysis of, 217, 221, 229, 242
artificial, preparation of, 210
collection of, 210
composition of, 201, 221
enzymes of, 201
human, collection of, 210
experiments on, 211
lactic acid in, test for, 238
origin of hydrochloric acid of, 203
Gastric juice, quantitative analysis of, 217, 229, 242
quantity of, 201
reaction of, 202
secretion of, 200, 202
influence of meat extractives on, 214
of psychical factors on, 215
of water on, 200, 210
specific gravity of, 201
Gastric lipase, 201, 206
Gastric protease, (see Pepsin)
Gastric rennin, 46, 201, **205,** 214,
action of, upon casein, 201, 205, 214, **533,** 540
experiments on, 214, 540
in gastric juice, absence of, 206
influence of, upon milk, 214, 540
nature of action of, 205, 533
occurrence of, 205
Gastric residuum, 221, 226
analysis of, 228
composition of, 221
removal of, 226
Gastrin, 201
Gelatin, 118, 119, 550, **551,** 552
decomposition of, 118, 119
experiments on, 552
influence of acid on the swelling of, 20
isoelectric point and precipitation of by alcohol, 20
solidification of, influence of acidity on, 21
Gels, diffusion through, 16
Gerhardt's test for acetoacetic acid, 659
Gerhardt's test for urobilin, 625
Gies' biuret reagent, preparation of, 165
Gigantism, 486
Gliadin, 118, 120, 158, 175
decompcsition of, 118
tests on, 177
Globin, 118, 120, 178, 336
decomposition of, 118
Globulins, 158, **173**
experiments on, 174
serum, 158, 332, 354, 647
vegetable, 174
Glucide, 66
Glucoproteins (see Glycoproteins)
Glucosamine, 178
Glucosazone, crystalline form of, Plate III, opposite p. 72
Glucose, 67, **69**
Benedict methods for determination of in urine, 744, 753, 754
Benedict's modification of Fehling's test, 76, 641
differentiation from lactose (Mathews), 89
experiments on, 71, 638
formulas for, 69, 70
glycosuria produced by, 865
hyperglycemia produced by, 863
in blood, determination of, 380–388
in finger-tip blood, determination of, 385
in urine, determination of by Sumner's method, 746
Power and Wilder's method, 749
official method, 751
optical activity of, 81
phosphate, 48
quantitative determination of, in blood, 381
in urine, 744
relative sweetness of, 71
tests on, 71, 638
tolerance tests, 388, 391
α-Glucose, 70
β-Glucose, 70
d-Glucose, 69
β-d-Glucose, 69
Glucosidases, 45
α-Glucosides, 45
β-Glucosides, 45
Glucosuria, renal, 383, 638
Glucothionic acid, 690
Glutamic acid, 118, 120, **136,** 139
formula for, 136
preparation of, 139
Glutamine, 299
Glutathione, 46, 53, 55
estimation of in tissue, 62
nitroprusside reaction for, 62
Glutelins, 158, 175
tests on, 176
Gluten, preparation of, 176
tests on, 176
Glutenin, 158, 175, 176
preparation of, 176
tests on, 176
Glycerol, 245, 246, 253
acrolein test on, 253
borax fusion test on, 253
experiments on, 253
formula for, 246
hypochlorite-orcinol reaction for, 253

Glycerol extract of pig's stomach, preparation of, 211
Glycerophosphatase, 46
Glycerophosphoric acid, 575, 593, 623
Glycine (see Glycocoll)
Glycocholic acid, 275
Glycocholic acid group, 275
Glycocoll, 118, 120, **128**, 275, 298
crystalline form of, 130, 282
formula for, 128, 275
free, preparation of, 130
from hippuric acid, preparation of, 129
preparation of, 130, 282
Glycogen, 67, 96, 560, **561**, 571
experiments on, 571
hydrolysis of, 571
in embryos, 561
iodine test on, 571
of muscular tissue, relationship of lactic acid to, 562
Glycogenase, 45
Glycolytic enzymes, 45
Glycoproteins, 159, **178**, 551
experiments on, 551
hydrolysis of, 551
Glycosuria, renal, 383, 638
Glycosuric acid, 619
Glycuresis, 621, 638
Glycuronates, conjugate, 300, **662**, 774
tests for, 662
Glycuronic acid, 300, **662**, 744
Glycyl-glycine, formation of, 171
Glycyl-tryptophane test, 266, **270**
Glyoxylic acid, 163
formula for, 163
reaction (Hopkins-Cole), 163
Gmelin's test for bile pigments, 279, 654
Rosenbach's modification of, 279, 654
Gold sol, preparation of, 15
Granular casts in urinary sediment, 691
Greenwald and Lewman's method for titratable alkali of blood, 440
Gregersen and Boas' variation of benzidine reaction, 349
Gross' method for quantitative determination of tryptic activity, 261
Growth, experiments on (see Dietary deficiencies and Vitamins)
Growth-promoting substances (see Vitamins)
Guaiac solution, preparation of, 885
Guaiac test on blood, 320, 342, 343, 347, 652, 653
on feces, 320, 322
on milk, 539
on pus, 653
on urine, 652
Guanase, 45
Guanine, 45, 190, 191, 192, 560, 568
Guanine chloride, crystalline form of, 197
Gum arabic, 67, 100
Gums and vegetable mucilage group of carbohydrates, 67
Gunning's iodoform test for acetone, 657
Günzberg's reagent, as indicator, 224
preparation of, 224, 885

Hagedorn and Jensen, method for determination of sugar, 385
Haine's test on sugar, 642
solution, preparation of, 885
Hair, human, 549
Hammerschlag's method for determination of specific gravity of blood, 345
Hanke and Koessler's *p*-diazobenzenesulphonic acid reactions, 303
Harington thyroxin formula, 482
Harris and Benedict, standards of, 507
Hart's casein method, 547
Häser's coefficient, 588, **710**
Hasselbalch equation, 426
Hay's test for bile acids, 280, 655
Heat of combustion in animals, 494
of combustion in a calorimeter, 494
Hektoen, Kretschmer and Welker, protein of, 649
Heintz method for determination of uric acid, 601
Heller's ring test for protein, 168, 644
Heller's test for blood in urine, 652
Hemagglutination, 347
Hemagglutinin, 336, 347
Hematin, 179, 336, 339, 352
Hematoidin, 276, 309, 312
crystalline form of, 276, 309
in urinary sediments, 687
Hematoporphyrin, 339, 582, 665
Hematoporphyrin, acid, 339, 417
alkaline, 339, 418
in urine, 582, 665
Hemicellulose, 67, 99
experiments on, 100
utilization of, by animals, 99
Hemin crystals, form of, 350
test, 349
Hemochromogen, 178, 339, 352, 354
crystalline form of, 352
Hemocyanin, 159, 179
Hemoglobin, 159, 178, 335, **336–340**, 351, 419
carbon monoxide, 339
colorimetric determination of, 454
crystalline form of, 337, 338, 351
decomposition of, 336, 339
derivatives of, relationship of, 339
diffusion of, 347
met, 339
oxy, 336, 337, 339, 351, 419
quantitative determination of, by acid hematin method, 455
reduced 339, 419
Hemoglobinometer, Newcomer, 454
Hemoglobins, 159, 178
Hemolysis, 331, 346
Herter's naphthaquinone reaction for indole, 303
Herter's para-dimethylaminobenzaldehyde reaction, 303
Heteroproteose, 160, 184
Hexone bases, 118, **121**
Hexosans, 67, 101
Hexosediphosphate, relationship to lactic acid formation, 563
Hexosephosphatase, 46
Hexoses, 67, **69**
Hildebrand's method for determination of hydrogen ion concentration, 30
Hippuric acid, 128, **612**, 614, 619, 859
experiments on, 613, 859
Griffith's method for quantitative determination of, 742
in urinary sediments, 686
synthesis of, 614, 859
demonstration of, 614, 859

Histamine, 201, 292, 294
formulas for, 292
Histidine, 118, 119, **151**
dihydrochloride, crystalline form of, 151
Histones, 158
Hoffmann's reaction for tyrosine, 150
Homogentisic acid, 618
Hopkins-Cole reaction, 163
on solids, 172
Hopkins-Cole reagent (Benedict modification), preparation of, 164, 886
Hopkins-Cole reagent, preparation of, 163, 885
Hordein, 158, 176
Hormone, female sex, 484
ovarian, preparation of, 485
Hormones, definition and discussion of, 255, 474–490
distinction from enzymes, 474
in blood, 255
Horn, composition of, 549
Human blood, composition of (table), 357
fat, composition of, 247
gastric juice, 221
characteristics of, 214
collection of, 210
hair, composition of, 549
milk, differentiation from cow's, 534–536, 539
Humoral stimulus, 201
Hunt method for activity of thyroid products, 482
Hunter and Givens' modification of Krüger and Schmidt's method, 739
Huppert-Cole test for bile pigments, 279, 654
Hyaline casts in urinary sediments, 691
Hydrobilirubin, detection of, in feces, 322
Hydrochloric acid solution, N/10, preparation of, (Reagents and Solution)
Hydrochloric acid of the gastric juice, 202, 221
origin of, theories as to, 203
seat of formation of, 201
Hydrogels, 3
Hydrogen acceptor, 54
electrode, electrometric titration of acids and bases using, 32
ion concentration, **8**, 12, 24, 27, 30, 448, 707, 709, 710
bicolorimetric method for, 707
chart, 12
colorimetric determination of, 24, 25, 448, 707
determination of by Hildebrand's method, 30
electrometric determination of, 27
titratable acidity and, 10, 26
of blood plasma, determination of (colorimetric), 448
electrometric, 451
of the urine, biocolorimetric methods, 709
as influenced by diet, 875
by acids, 878
by alkali, 878
determination of, 707–710
electrometric method, 710
Hydrogenated fat, 246
Hydrolysis of sucrose, 90, 91
Hydrosols, 3
Hydroxy, aromatic acids,tests for, 303
glutamic acid, 120, **139**
β-Hydroxybutyric acid, 357, 637, **656–662, 758–763**
Hydroxyproline, 118, 119, **141**
Hyperacidity, 202, 217
curve, 217
Hyperglycemia, 863
produced by carbohydrate ingestion, 863
Hypersecretion, acidity curve in, 231
Hyperthyroidism, 501
Hypothyroidism, 503
Hypoacidity, 202
Hypobromite solution, preparation of, 886
Hypochlorite-orcinol reaction for glycerol, 253
Hypoxanthine, 192, 560, 568, 572
chloride, crystalline form of, 573
formula for, 192, 568
oxidase, 192
silver nitrate, crystalline form of, 573

Icteric index, 396
Imbibition, 4
Imidazoles, phenols and hydroxy aromatic acids, tests for, 303
Indican, 301, 611, 765
Parker's method for determination of, 765
tests for, 611
Indicator solutions, preparation of, (see Reagents and Solutions)
standards, preparation of, 709
Indicators, chart of, 12
experiments on, 10–15, 221–224
pH range of, 222
table of, 222
tabulation of results of tests on, 223
use of, 10–15, 221
in gastric analysis, 21
Indole, 294, 303
formula for, 294
in feces, quantitative determination of, by Bergeim's method, 328
origin of, 294
tests for, 303
Indoxyl, 301, 610
formula for, 301, 612
origin of, 301
Indoxyl-potassium sulphate (see Indican)
Indoxyl-sulphuric acid, 610
formula for, 610
Infraproteins (see Metaproteins)
Inorganic catalysts, experiment on, 55
matter deficiency, influence of, 845
metabolism, experiments in, 868
phosphate, Benedict and Theis method for determination of, 406
determination of, in blood, 403
sulphate in blood, determination of, 406
Insulin, 46, 477
crystalline form of, 478
effect of on blood sugar, 479
experiments on, 478
molecular weight of, 478
preparation of, 478
unit of, 478
Insurance, life, method for albumin in urine, 756
glucose in urine, 751
Intarvin, 247
Intensity factor, 8
Intestinal digestion, 266
juice, 266
enzymes of, 45, 266, 267, 268–272
preparation of, 268–272

Inulase, 45, 95
Inulin, 67, 95
  experiments on, 95
Inversion, 90, 91
Invert sugar, 71, 90
  relative sweetness of, 71
Invertase (see Sucrase)
Invertases, experiments on, 270
Invertin (see Sucrase)
Inverting enzymes, 45, 266, 270
Iodide of dextrin, 96
  of starch, 93
Iodine absorption test, 253
  deficiency, 847
  in blood and in animal tissues, method of Kendall and Richardson for determination of, 483
  solution N/10, 886
  test for dextrin, 96
    starch, 93
    urobilin, 625
Iodine-zinc-chloride reaction, 99
Iodoform test for alcohol, 79
Iodo-thyreoglobulin, 479, 482
  preparation of, 482
Iron, in blood, 336, 346
  detection of, 346
  in bone ash, 557
  detection of, 557
Iron deficiency, 847
  in foods, Walker's method for determination of, 413
  in protein, 113
  in urine, 636, 788
  detection of, 636
  determination of, 788
Irradiation, ultraviolet, 824
Isoelectric point, 6
  and precipitation of gelatin by alcohol, 20
  and solubility of casein, 19
  state, 6
Isohydric change, 420
Isoleucine, 115, **134**
Isomaltose, 67, 88

Jaffe's reaction for creatinine, 609
  chemistry of, 609
Jaffe's test for indican, 611
Jolles' reaction for indican, 612
Juice, gastric, 203, 210
  intestinal, 266
  pancreatic, 255

Kafirin, 177
Kantor and Gies's biuret paper, 165
Kapsinow test for bile pigments, 655
Karr's method for determination of blood urea by direct nesslerization, 373
  salt mixture, 801
Kaolin, use of, 167
Kastles peroxidase reaction, 539
Kendall's method for the determination of iodine in thyroid tissue, 483
  in blood and in animal tissues, 483
  thyroxin crystals, 481
  formula, 481
Kephyr, 89
Keratin, 158, 177, 549
Keto form of uric acid, 600
Ketogenesis, 873
Ketogenic balance, 874
Ketogenic-antiketogenic ratio, 874
Ketone, 66, 74
Ketonuria, 872
Ketose, 66
Ketosis, **872**
Kidney efficiency tests, 675
Killian, carbohydrate tolerance test, 388
Kjeldahl method for determination of nitrogen, 711
Kjeldahl-Folin-Farmer nitrogen method, 714
Kjeldahl-Folin-Wright nitrogen method, 713
Kober nephelometer-colorimeter, 364
Koch and McMeekin's method for direct nesslerization of nitrogen of blood, 370
  urine, 717
Koumyss, 89
Kramer and Gittleman's method for determination of sodium in serum, 411
Kramer and Tisdall's method for determination of potassium in serum, 412
Krüger and Schmidt's method for the quantitative determination of purine bases, 738
  of uric acid, 738
Kynurenic acid, 619

Laccase, 45, 59
Lactalbumin, 118, 158
  decomposition products of, 118
  quantitative determination of, 547
Lactase, 45, 266, **267**, 271
  experiments on, 271
Lactic acid, 89, 203, 225, 238, 357, 531, **561–563**, **743**
  Clausen's method for determination of in urine, 743
  in blood, 400
  fermentation, 89
  formation, relationship to hexosediphosphate, 563
  in muscular tissue, 561
  of muscle, relation of glycogen to, 562
  production of during muscular contraction, 560
  in stomach contents, 225, 238
  in tissues, determination of, 401
  relationship of to musuclar contraction, 560, 562
  tests for, 238
Lactosazone, crystalline form of, Plate III, opposite p. 72
Lactose, 67, **88**, 530, 535, **536**, 542, 548
  differentiation from galactose, 89, 90, 665, **666**
  experiments on, 88, 665
  fermentation of, 89
  in urine, 665
  quantitative determination of, 548
  relative sweetness of, 71
Langerhans, islets of, 477
Lanolin, 248, 259
Lead acetate, basic, preparation of, 883
Leach's hydrochloric acid test for formaldehyde, 542
Lecithin, 159, 179
  experiments on, 578
Lecithoproteins, 159, 179

Leucine, 118, 120, **133**
experiments on, 134
in urinary sediments, 686
Leucocytes, 331, **340**
Leucocytosis, 341
Leucosin, 167
Levo–α-proline, crystalline form of, 140
Levulosazone, crystalline form of, Plate III, opposite p. 72
Levulose (see Fructose)
Lewis and Benedict's method for sugar in blood, Benedict's modification of, 387
Lichenin, 67, **96**
Leiberkühn's jelly (see Alkali metaprotein)
Liebermann-Burchard test for cholesterol, 281, 579
Life insurance method for albumin in urine, 756
glucose in urine, 751
Lime water, preparation of, 886
Line test for calcium-depositing substances, 838
Linoleic acid, 246
Lipase, gastric, 201, 206
Lipase, pancreatic, 45, 58, 256, 258, 264
copper soap test for, 264
ethyl-butyrate test for, 264
experiments on, 58, 264
influence of bile on, 264
litmus-milk test for, 264
Lipases, 45, 58, 206, 256, 258, 264
autolytic, 45
experiments on, 58, 264
pancreatic, 58, 256, 258, 264
vegetable, 45, 58
Lipeses, 49
Lipins, 575
Lipoid phosphorus (Lecithin) in blood, determination of, 395
Lipoids of nervous tissue, 575, 578
Lipolytic enzymes (see Lipases)
Litmus milk, powder, 887
"Litmus-milk" test for pancreatic lipase, 264
Logarithm tables, 903–4
Long's coefficient, 588, **710**
Lugol's solution, preparation of, 887
Lyle-Curtman guaiac "procedure," 320, 347
reagent, 321, 347
Lymph, 344
Lysine, 118, 119, **145**, 842
deficiency, demonstration of, 842
Lysine picrate, crystalline form of, 146

MacLean and de Wesselow, urea concentration test for kidney efficiency, 678
Magnesia mixture, preparation of, 887
Magnesium balance, preparation of, 870
in bone, detection of, 556
in urine, 634, 784
quantitative determination of, 784
nitrate solution, 887
in serum, Denis method for determination of, 410
Maefatti's formol titration method for ammonia in urine, 727
Maltase, 45, 266, 267, 272
experiments on, 272
Maltosazone, crystalline form of, Plate III, opposite p. 72
Maltose, 67, **87**
experiments on 88
Maltose, relative sweetness of, 71
structure of, 88
Manometer, Barcroft, 51
Manometric determination of carbon dioxide in blood or plasma, 464
carbon monoxide in blood, 468
methemoglobin in blood, 469
oxygen in blood, 466
plasma carbon dioxide capacity, 465
urea in blood, 470
urea in urine, 470
methods of analysis of gases in blood and other solutions, 458
Marriott method for alkali reserve, 443
Marshall's clinical urease method for estimation of urea in urine, 722
McCollum's salt mixture, 809
rachitic diet No. 3143, 835
McCrudden's method for determination of calcium, 784
of magnesium, 784
Meeker and Oser's electrometric titration method for hydrogen ion concentration, 34, 36, 451
Melting-point apparatus, 253
of fats, determination of, 253
Mendel and Osborne, salt mixture, 809, **831**
growth curves, 832
Menstrual blood, 342
Mercury in urine, 670
detection of, 670
Metabolic balance, preparation of, 870
product nitrogen, 314, 862
rate, basal, 509
Metabolism, 491, 789
acid-base, experiments on, 872
basal, 491, 500
energy, 491
experiments, 491, **789**
balance of income and outgo in, preparation of, 870
collection and preservation of feces in, 790
on dogs, Cowgill's procedure for, 799
separation of feces in, 325, 791
urine in, 789
Folin's theory of, 861
influence of digestion on, 862
of fats and carbohydrates as protein sparers in, 851
of high, calorie, non-nitrogenous diet on, 852
of indigestible, non-nitrogenous material on, 792
of ultraviolet light on, 829
of water on, 871
of acid-forming and base-forming foods, 875
of ammonium benzoate, 859
of carbohydrates, 831, **863**
of energy, 831, **845**
of fat, 831, **868**,
of inorganic elements, 831, **868**
of nitrogen, 849
of nitrogen and sulphur as influenced by diet, 860
of proteins, 831, **849–851**, 860
time relations of, 849
of purines, 855–858
on "salt-free" diet, 868
on salt-rich diet, 869

Metabolism, manipultory procedures, 789
protein, 495
relation of bacterial nitrogen of feces to, 861
of metabolic product nitrogen of feces to, 862
reference books and reviews, 492
respiratory and neutrality regulation, 419
studies in, 789
study of creatinine elimination in, 859
tables, 894–902
time relations of protein, 849
Metaproteins, 160, **180**
acid, 160, 180
experiments on, 180
alkali, 160, 180, 181
experiments on, 181
precipitation of, 181
sulphur content of, 181
Methemoglobin, 339
in blood, manometric determination of, 469
Methyl orange, use of, as indicator, 12, 222, 887
red, use of, as indicator, 12, 222, 887
Methyl-pentose (see Rhamnose)
Methylphenylfructosazone, formation of, 84
Methylphenylhydrazine, 84
Mett's method for determination of peptic activity, 234
Mett's tubes, preparation of, 235
Meulengracht's icteric index, determination of, 396
Mice, white, as experimental animals, 802
Micro-organisms in urinary sediments, 697
in feces, 291, 308, 311, **313**, 327, 861
in intestine, **291**, 308, 311, 313, 327, 861
Microrespirometer, illustration of, 51
Milk, 530
ash of human and cow's, 546
calcium phosphate from, 542
casein of, 530, 533, 535, **537**, 541
collection of human, for analysis, 543
composition of, in relation to rate of growth of young, 536
composition of human and cow's, 535
constituents of, 530, 531
curds, photographs of, 533, 534
detection of calcium phosphate in, 541
lactose in, 542
preservatives in, 542
dietary efficiency of 530, **848**
difference between human and cow's, 534–536, 539
dried, cow's, 536
human, 536
human in infant feeding, 536
experiments on, 538
human and cow's, differentiation, 534, 535
lactose in, 530, 531, 536, 537
crystalline form of, 537
fermentation of, 537
powder, 531
preparation of casein from, 541
properties of casein of, 532, 533, 537, 541
quantitative analysis of, 543
raw and heated, differentiation of, 539
reaction of, 531, 538
separation of coagulable proteins of, 546
serum, 531
souring of, 531
specific gravity of, 532, 539
Milk, summer and winter, 531
variation in composition of, during different periods of lactation, 535
vitamines in, 530
Millon's reaction, 162
reagent, preparation of, 163, 887
Molecular weights, 114
Molisch's reaction, 71
reagent, 887
Molybdate solution, preparation of, 887
Monavitaminosis, 803
Monosaccharides, 67, **69**
Barfoed's test for, 77
classification of, 67
Mörner's reagent, preparation of, 150
test for tyrosine, 150
Mosenthal test for kidney function, 676
Mucic acid, 85, 89, 665, 666
test, 85, 89, 665, 666
Mucin, 105, 108, 109, 159, **178**
biuret test on, 109
hydrolysis of, 109
isolation of, from saliva, 109
Millon's reaction on, 109
Mucins, 159, 178
experiments on, 551
hydrolysis of, 551
in urine, 650
preparation of, from tendon, 551
Mucoids, 159, **178**, 550, 551, **650**
Murexide test, 604
Muscle plasma, 559, 568
Muscular contraction, production of lactic acid during, 560
relationship of to lactic acid, 562
tissue, 559
ash of, smooth and striated, 566, 567
commercial extracts of, 567
experiments on "dead," 570
"living," 568
pigment of, 567
relationship of lactic acid to glycogen, 562
rigor mortis of, 559, 560
separation of extractives from, 572
Mutarotation, 70, 82
Myers and Wardell, determination of cholesterol in blood, 391
Myers' bicolorimeter, 26, 362
colorimeter, 360
direct aeration method for urea in blood, 374
Myosin, 559, 570
experiments on, 570
Myosinogen, 559
Myxedema, effect of thyroxin on, 480

α-Naphthol reaction, 71
solution, 71, 887
Naphthoresorcinol reaction for glycuronates (Tollens), 662
Nephelometer, Bloor, cut of, 364
description of, 364
Nephelometry, 363
Nervous tissue, 575
experiments on, 578
Nessler's solution, 887
Neutral fats, 246, 247, 250
Neutral olive oil, preparation of, 251, 888
Neutral red, use of, 888
Neutrality regulation, 419

Newcomer hemoglobinometer, 454
plate, standard, 454, 456
Ninhydrin, formula for, 124
Ninhydrin reaction, 166
Nitric acid test (Heller), 168, 644
Nitric acid-$MgSO_4$ test (Roberts), 168, 646
Nitrilase, 49
Nitrilese, 49
Nitrites in saliva, test for, 109
Nitrogen, 113, 114, 115
forms of, in protein molecule, 114
importance of, in sustaining life, 114
in urine, quantitative determination of, 711
metabolism, experiments in, 849
Nitrogen distribution, calculation of, 712
Nitrogen "lag," 849
metabolic product, 862
"partition," 860
Nitroprusside test for creatinine (Weyl), 609
Nitroprusside-acetic acid test for creatinine (Salkowski), 609
Non-blood proteins, 648
Non-protein nitrogen of blood, determination of 368
Koch-McMeekin method for, 370
Normal urine, 581, 592
characteristics of, 581
constituents of, 592
experiments on, 595–636
Nucleases, 45
Nucleic acid, 159, 188, 189, 195–198
decomposition of, 189–191
experiments on, 195–198
from yeast, formula for, 190
Nucleicacidase, 45, 191
Nucleins, 188, 189
Nucleoproteins, 159, 179, 188, 189, 194, 195, 575, 650
decomposition of, 188, 189
experiments on, 194, 195
from yeast, 194
preparation of, 194
protein, carbohydrate and phosphoric radicals in, 190, 191
tests on, 194
thymus, preparation of, 195
experiments on, 195
Nucleosidase, 45, 191
Nucleoside, 191
Nucleotidase, 191
Nucleotide, 190
Nucoa, vitamin content of, 805
Nutrition, adequate vs. optimal, 848
Nylander reaction, 77, 642
Nylander reagent, preparation of, 77, 888

Obermayer's test for indican, 612
reagent, preparation of, 612, 888
"Occult" blood in feces, 312, 320
tests for, 320
Olein, 246, 247
Olive oil, 250
emulsification of, 251
neutral, preparation of, 251, 888
Opthalmia, 803
Optical methods, 80–83
Orcinol-HCl reaction (Bial), 86, 664
Organic acid, determination of, 706
Organized urinary sediments, 668
Ornithine, 126, 300
Ortho-tolidin test for blood, 239, 321, 348, 652
Osborne and Mendel's salt mixture, 809, 831
growth curves, 832
Osborne-Folin method for determination of total sulphur in urine, 773
Osmotic pressure, 2, 7
demonstration of, 18
of colloidal solutions, demonstration of, 18
of a true solution, 17
Osseoalbumoid, 555
Osseomucoid, 159, 178, 555
chemical composition of, 178
Osseous tissue, 555
experiment on, 556
Ostwald viscosimeter, estimation of viscosity with, 17
Ott's precipitation test for detection of nucleoprotein in urine, 650
Ovalbumin, 158
Ovarian hormone, preparation of, 485
rat unit of, 485
Ovoglobulin, 158
Ovovitellin, 159, 160, 178
Oxalated plasma, preparation of, 354
Oxalic acid, 615, 769
experiments on, 615
quantitative determination of, 769
standard solution of, preparation of, (Reagents and solutions)
Oxaluria, 615
Oxidase system, 53
Oxidases, 45, 59, 538
experiments on, 59
Oxidation, 297
and reduction systems, 51
of carbohydrates, 494
of fats, 495
β-Oxidation, 247
Oxidations, study of, 61
Oxyacids, 303, 618
β-Oxybutyric acid (see β-hydroxybutyric acid)
Oxycalorimeter of Benedict and Fox, 853
Oxygen in blood, determination of, 451
capacity, determination of, 451
in blood, manometric determination of, 466
Oxygenase, 53
Oxyhemoglobin, 336, 337, 339, 351, 419
crystalline forms of, 337, 338, 351
Reichert's method for crystallization of, 351
Oxyproline (see Hydroxyproline)

Palmitic acid, 245, 246, 252
crystalline form of, 252
experiments on, 252
formula for, 246
preparation of, 251
Palmitin, 246
Pancreas, 473, 477
Pancreatic amylase, 45, 56, 256, 262
digestion of raw starch by, 258, 262
inulin by, 262
experiments on, 56, 262
influence of bile upon action of, 262
most favorable temperature for action of, 262
Pancreatic digestion, 255
general experiments on, 260
products of, 256, 259

Pancreatic insufficiency, Schmidt's nuclei test for, 323
Pancreatic juice, **255**, 259
artificial, preparation of, 259
daily secretion of, 255
enzymes of, 255
freezing-point of, 256
mechanism of, secretion of, 255
reaction of, 256
solid content of, 256
specific gravity of, 256
Pancreatic lipase, 45, 58, 256, 258, 264
copper soap test for, 264
ethyl-butyrate test for, 264
experiments on, 58, 264
litmus-milk test for, 264
Pancreatic protease (see Trypsin)
Pancreatic rennin, 45, 259, 264
experiments on, 264
Pappenheimer, McCann and Zucker's salt mixture, 837
rachitic diet No. D, 837
Paracasein, 46,
soluble, 205
Parahormones, 474
Paralactic acid, 333, **561**, 593, **623**
Paraoxy-β-phenyl-α-amino-propionic acid, 120, 148
Parasites, 308, 311, 315, 697
Parathyroid extract, effect of, 489
preparation of an active, 490
unit, 489
glands, 473, 488
tetany, 489
Parathyroids, 473, 488
experiments on, 490
Parker's method for determination of indican, 765
Pathological urine, 581, 637
constituents of, 637
experiments on, 638
"Partition" of nitrogen and sulphur in urine, 860
Pellagra-like condition in albino rat, 816
Pellagra-preventive substance, 815
Pellagrins, 815
Pentamethylenediamine, 292
Pentosans, 67, 99, 100
Pentosazone, crystals of, 663
Pentoses, 67, 85
experiments on, 86
in urine, 663
tests for, 664
Pepsin, 45, 57, **203**, 211, 234
action of, influence of bile upon, 213
influence of different acids upon, 212
influence of metallic salts upon, 213
temperature upon, 212
conditions essential for action of, 204, 211
differentiation of, from pepsinogen, 203, 212
digestive properties of, 204
formation of, 203
most favorable acidity for action of, 212
presence of, in intestine, 205
proteolytic action of, 203
Pepsin-hydrochloric acid, 211
Pepsinogen, 47, 203, 212
differentiation of, from pepsin, 203, 212
extract of, preparation of, 210, 211
formation of, 203
Pepsin-rennin controversy, 206
Peptases, 45
Peptic activity, Given's modifications of Rose's method for determination of, 236
Mett's method for the determination of, 234
Rose's method for determination of, 235
Peptic and tryptic activity, reversibility of, 49
Peptic proteolysis, 203
products of, 204
relation of, to tryptic proteolysis, 204
Peptides, 116, 160, 185
Peptone, 115, 160, 183
differentiation of, from proteoses, 184
experiments on, 184
in urine, 644, 648
separation of, from proteoses, 184
Permutit, 888
use of in determining ammonia, 727
Peroxidase, 52
reaction, Kastle's, for milk, 539
Peroxidases, 45, 52, 539
Peters' method for sugar determination, 747
Pettenkofer's test for bile acids, 279, 655
Mylius's modification of, 279, 655
Phenol, 293, 296, 303, 400, 611, 766
excretion of, 611, 766
quantitative determination of, in urine, 766
in blood, 400
tests for, 303
Phenol potassium sulphate, 301
Phenolphthalein as indicator, 12, 222, 224
preparation of, 889
tests for blood in feces, 321
Phenols, hydroxy aromatic acids and imidazoles, test for, 303
Phenolsulphonephthalein test for kidney efficiency, 675
Phenylacetic acid, 296, 299
Phenylacetylglutamine, preparation of, 304
Phenylalanine, 118, 120, **147**, 292
Phenylethylamine, 292
formula for, 292
Phenylglucosazone, Plate III, opposite p. 72
Phenylhydrazine, 72
acetate solution, preparation of 73, 889
mixture, preparation of, 72, 889
reaction, 72
Phenyllactosazone, crystalline form of, Plate III, opposite p. 72
Phenylmaltosazone, crystalline form of Plate III, opposite p. 72
Phenylpropionic acid, 293
Phosphate, total, Fiske and Subarrow's method, 776
inorganic, determination of in blood, 403, 406
mixtures (phenol red range), 449
Phosphatase, 49
Phosphates in urine, 631, 633, 776
detection of, 633
experiments on, 633
quantitative determination of, 776
Phosphatese, 49
Phosphatides, 274, 531, 577
Phosphonuclease (nucleotidase), 268
Phosphoproteins, 159, 160, **178**, 537
Phosphoric acid, 631
Phosphorus, total acid-soluble in blood, determination of, 405
Phosphorus in urine, determination of, 776
organic, test for, 194

Photocatalyst, chlorophyll as, 68
Photosynthesis, 67
Physical chemistry, 1
Physiological constituents of urine, 592
Phytase, 46
Phytin, 46
Picric acid reaction for creatinine (Jaffe), 609
method for sugar in blood (Benedict), 387
purification of, 889
saturated solution, 891
test for glucose, 78
Pigments of urine, 582, **624**
Pineal gland, 473, 488
Piria's test for tyrosine, 150
Pituitary gland, 473, 486
Plasma bicarbonate, titration method of Van Slyke, 437
of blood, 331, 354
carbon dioxide capacity, manometric determination of, 465
of muscle, 559, 568
Plasmaphæresis, 335
Polariscope, use of, 80
Polyavitaminosis, 803
Polypetides, 116, 121, 161, 185
Polysaccharides, 67, 91
classification of, 67
properties of, 91
Posner's modification of biuret test, 166
Potassium hydroxide test for pus in urine(Donné), 653
permanganate, standard solution, preparation of, 891
in serum, Kramer and Tisdall's method for determination of, 412
in urine, 634, 786
quantitative determination of, 786
Power and Wilder's method for determination of glucose in urine, 749
P-P factor, 803, 804, **815**
sources of, 804, 815
Primary protein derivatives, 159, **179**
Primary proteoses, 185
Products of protein hydrolysis, 115, 118, 119, 120, 121, 256
Prolamins, 158, **176**
classification of, 158
preparation of, 177
tests on, 177
Proline, 115, 118, 119, **140**, 176, 204, 256
crystalline form of copper salt of, 141
crystalline form of lævo-α-, 140
Prosecretin, 255, 474
Protamines, classification of, 159, 160
Proteans, 159, **179**
Protease, gastric, 46, 57, 203, 211
experiments on, 57, 211
pancreatic, 46, 57, 256
experiments on, 57, 259
vegetable, 46, 58
Proteases, 46, 57
experiments on, 57
Protective colloid, demonstration of, 16
Proteid (see Protein)
Protein, Bence-Jones, crystalline form of, 648
content of foods, 850
deficiency, influence of, 838
derivatives, primary, 159, 179
Protein derivatives in urine, Folin's gravimetric method for the determination of, 754
secondary, 160, 179, **183**
of Hektoen, Kretschmer and Welker, 649
metabolism, 495
influence of water on, 871
time relations of, 849
utilization, determination of, 851
Protein-coagulating enzymes, 46, 205, 259, 341 533
Protein-sparing action of fat and carbohydrate, 851
Proteins, **113, 157**
acetic acid and potassium ferrocyanide test for, 168
action of alkaloidal reagents on, 168
of metallic salts on, 167
mineral acids, alkalies and organic acids on, 167
biuret test on, 164
chart for use in review of, 186
chemical composition of, 113
classification of, 158, 160
coagulated, 160, 181
quantitative determination of, 754
tests on, 183
coagulation, influence of salts upon, 182
coagulation or boiling test for, 169
color reactions of, 162
conjugated, 159, **177**, 188
classes of, 159, 177
experiments on, 109, 194, 351, 541, **551**
nomenclature of, 159, 177
occurrence of, 159, 177
decomposition of, **113**, 115, 118, 119, 120, 256
by hydrolysis, 115, 118, 119, 120, 256
by oxidation, 115
products of, 115, 161, 256
experiments on, 113
study of, 113
derived, 159, **179**
formation of fat from, 248
formulas of, 114
Heller's ring test on, 168
Hopkins-Cole reaction on, 163
importance of, to life, 113
in blood, determination of, 398
in urine, **643**, 754
determination of, 754
tests for, 644–650
Millon's reaction on, 162
molecular weights of, **114**
ninhydrin reaction on, 166
non-blood, 648
precipitation reactions of, 166
quantitative determination of, in milk, 546
review of, 185
Robert's ring test on, 168
salting-out experiments on, 169
salts of, 166
scheme for detection of, 187
simple, 158, 160, **161**
synthesis of, 49, **113**
xanthoproteic reaction on, 163
Proteolysis, peptic, 204
tryptic, 204, 256
Proteolytic enzymes (see Proteases)
Proteose, 115, 160, 161, 183
experiments on, 184, 185

Proteose in urine, 648
test for, 649
powder, preparation of, 185
Schulte's method for detection of, 649
separation of, from peptones, 184
Proteoses and peptones, 183
experiments on, 184, 185
Prothrombin, 341
Pseudoglobulin, 331, 332
Psychical stimulation of gastric secretion, 200
Ptyalin (see Salivary amylase)
Puffer fish oil, 248
Purinases, 46, 192
Purine bases, **191**, 195, 196, 198, 626, 738, 739
formulas for, 192, 568
tests on, 196
content of foods, 857
excretion, rate of, 858
oxidases, 45, 192
in urine, quantitative determination of, 738
Purine-free and high purine diets, influence of, 855
Purines, amino, 192, 198
oxy, 192, 196, 197
Pus casts in urinary sediments, 695
Pus cells in urinary sediments, 689
in urine, 653
tests for, 653
Putrefaction and detoxication, **291**
detoxication and reduction, experiments on, 303
formula for, 292
indican as an index of, 296, 610, 765
products, 291
experiments on, 303
tests for, 303
Putrescine, 292, 295
Pyrimidine bases, 193, 199
experiments on, 199
formulas for, 193
α-pyrrolidine-carboxylic acid (see Proline)
Pyuria, 653

Qualitative analysis of the products of salivary digestion, 110
Quantitative analysis of blood, 356
of expired air, 499
of gastric juice, 229
of milk, 543
of urine, 702
Quantity factor, 8

Rachitic diets, 835
Raffinose, 67, 91
relative sweetness of, 71
Rancid fat, 247, 250
Rats, white, experiments on, (see Albino rats), 828–849
Reagents and solutions, **882**
Reduction, 297
and oxidation systems, 51
putrefaction and detoxication, experiments on, 303
Regurgitation, automatic regulation of gastric acidity by, 220
Rehfuss stomach tube, illustration of, 218
use of, 218, 225
Reichert's method for crystallization of oxyhemoglobin, 351
Reinsch test for arsenic, 670
for mercury, 670
Renal diabetes, 383
glycosuria, 383
Rennin, gastric, 46, **205**, 214, 533, 540
experiments on, 214, 540
Rennin pancreatic, 46, 259, 264
experiments on, 264
Reproductive vitamin, (see Vitamin E)
Resorcinol-HCl reaction, 84, 667
Respiration, 497
calorimeter, Atwater-Rosa-Benedict, 510
Benedict-Collins, 513
Benedict portable, 512
Benedict-Roth, 513
Benedict student, 515
universal, 511
chemistry of, 419, 431, **497**
external, 497, 498
internal, 497
Respiratory exchange, determination of, 491
metabolism and neutrality regulation, 419
quotient, 52, **495**
Retention meal in gastric analysis, 228
Reticulin, 177
Reversibility of enzyme action, 49, 108
of peptic and tryptic activity, 49
Rhamnose, 67, 87
relative sweetness of, 71
Ricin, 58, 347
Rickets, 803, 804, **820**, 835, 838
active, radiograph of, 837
healed, radiograph of, 837
influence of ultraviolet light in, 829
"line test" for, 838
Rigor mortis, 559, 560
Ring method, determination of surface tension by, 23
Robert's ring test for protein, 168, 646
reagent, preparation of, 168, 891
Rosenheim-Drummond, color reactions for vitamin A, 810
volumetric methods for sulphates and total sulphur, Fiske's modification of, 774
Rose's method for determination of pepsin, 235
Ruttan and Hardisty's ortho-tolidin test for blood, 239, 321, 348, 652
Ryle's gastric tube, illustration of, 219

Saccharose (see Sucrose)
Sahli's desmoid reaction, 213
reagent, 231, 891
Saliva, 104
experiments on, 108
Salivary amylase, 41, 45, 55, 106, 202, 258
activity of, in stomach, 107, 202, 258
inhibition of activity of, 107
nature of action of, 106
products of action of, 106, 110
scheme showing, 106
Salivary digestion, 104
experiments on, 110
graphic representation of, 106
in stomach, 107, 202, 258
Salkowski's test for cholesterol, 281, 579
for creatinine, 609
Salmine, 118, 119, 159
Salt mixture, Cowgill s, 801
Karr's, 801

Salt mixture, McCollum's, 809
Osborne and Mendel's, 831
Pappenheimer, McCann and Zuckers, 837
"Salt-free" diet, metabolism on a, 868
Saponification, 245, 251, 252, 285
of bayberry tallow, 251
of lard, 252
Sarcolactic acid, 561
Schaffer and Marriott method for acetone bodies, 762
Schardinger reaction, 61
Scheme for analysis of biliary calculi, 280
bone ash, 557
stomach contents, 225
urinary calculi, 700
detection of carbohydrates, 103
of proteins, 187
Schiff's reaction for uric acid, 605
Schmidt diet, composition of, 315
Schmidt's nuclei test for pancreatic insufficiency, 323
Schmidt's test for hydrobilirubin, 322
Schütz-Borissow law, statement of, 50, 234
Schweitzer's reagent, action of, on cellulose, 99
preparation of, 891
Scleroproteins (see Albuminoids)
Scombrine, 118, 159
Scombrone, 159
Scurvy, 803, 816
Secondary protein derivatives, 183
Secretin, 255, 474
Seliwanoff's reaction, 84, 667
reagent, preparation of, 84, 667, 891
Separation of feces, importance of, in nutrition and metabolism experiments, 325, 791
Serine, 115, 118, 120, **132**
crystalline form of, 132
formula for, 132
Serum albumin, 158, 162, 331, 333, 354, 643, 644
crystalline form of, 171
in urine, 643, 644
test for, 644
Serum, blood, 333, 353
Serum globulin, 158, 174, 332, 643, 647
in urine, 643, 647
tests for, 647
Sex hormone, female, 484
Shohl and Pedley's method for determination of calcium in urine, 784
Skatole, 155, 156, 294, 296, 301, 303
tests for, 303
Slow stomach, evacuation of food by, 209
Soap, salting-out of, 252
insoluble, preparation of, 252
Sodium and potassium in urine, 634, 786
quantitative determination of, 786
Sodium alcoholate, N/10 solution, 891
in serum, Kramer and Gittleman's method for determination of, 411
Sodium alizarin sulphonate, preparation of, 891
Sodium chloride in urine, 630, 780
Sodium hydroxide solution, N/10, preparation of, 703
saturated solution, preparation of, 891
Sodium hypobromite solution, preparation of, 886
Sodium thiosulphate, standard solution, preparation of, 892
Sodium tungstate solution, 892
Solera's reaction for detection of thiocyanate in saliva, 109
test paper, preparation of, 110
Sols, 3
Soluble starch, 55, **92**, 106
as indicator, 232,
Solutions and reagents, 882
Sørensen's formol titration method for amino nitrogen, 728
indicator method for hydrogen ion concentration, 12
Soxhlet apparatus for extraction of fat, 546
Specific conductance, 40
Specific rotations of carbohydrates, 81
Specificity of enzyme action, 48
Spectroscope, use of, in detection of blood, 414
Spermatozoa in urinary sediments, 696
microscopical appearance of human, 696
Stalagmometer, Traube, 22
Standard Newcomer Plate, 454, 456
Starch, 67, **91**
dry, action of iodine on, 93
experiments on, 92
hyperglycemia produced by 863
microscopical characteristics of, 92
potato, preparation of, 92
raw, digestion of, by pancreatic amylase, 262
raw, digestion of, by salivary amylase, 110
soluble, 55, 92, 106, 232
various forms of, 94
Starch paste, digestion of, by pancreatic, amylase, 56, 262
by salivary amylase, 55, 106, 110
experiments on, 93–95
preparation of, 93, 892
Steapsin (see Pancreatic lipase)
Stearic acid, 246, 576
Stearin, 246, 247
Steenbock rachitic diet No. 2965, 835
Stehle's gasometric method for urea, 723
Stercobilin, 309
Sterility disease, 803, **819**
Stockholm and Koch method for total sulphur in biological material, 407
Stokes' reagent, preparation of, 305
Stomach contents, examination of, 229
removal of, 229
tube Rehfuss, 218, 225
Ryle, 218, 219
Stomachs, fast, slow, 209
Sturine, 118, 119, 159
Sublingual glands, characteristics of saliva secreted by, 104
Submaxillary glands, characteristics of saliva secreted by, 104
Substrate, 43
Sucrase, 45, 59, 266, 270
experiments on, 59, 270
relative sweetness of, 71
vegetable, 59
Sucrose, 67, 90
experiments on, 91
inversion of, 90
structure of, 90
Sucrose- H_2SO_4 test (Pettenkofer), 279, 655
Sugar (see Glucose and Sucrose)
Sugar in normal urine, determination of, 753
Sugars, relative sweetness of, 71
Sullivan's qualitative test for cysteine, 145

Sulphate, total, in blood, determination of by Denis and Leche's method, 407
inorganic, in blood, determination of by Denis method, 406
Sulphates, in urine, 628, 770, 774
ethereal, 628
quantitative determination of, 771, 774
experiments on, 629
inorganic, 628
quantitative determination of, 771, 774
total, quantitative determination of, 770, 775
Sulphocyanides (see Thiocyanates)
Sulphur in protein, 113, 172
acid, 173
in urine, gravimetric determination of, 772
volumetric determination of, 774
lead blackening, 173
loosely combined, 173, 628
mercaptan, 173
neutral, 628, 775
oxidized, 173, 628
"partition," in urine, 860
tests for, 173
unoxidized, 173, 628
Sulphuric acid test (Piria), 150
Sulphuric acid N/12 and 2N/3, preparation of, 892
Sumner's method for determination of glucose in urine, 746
Suprarenal glands (see Adrenal glands)
Surface tension, 1, 7
demonstration of, 22
determination of relative, by the drop method, 22
determination of by the ring method, 23
by the drop method, 22
by the ring method, 23
Suspensoids, 3
Synthesis of hippuric acid, 614, 859
demonstration of, 859

Table of atomic weights, 905
Tables for use in metabolism determinations, 894–902
Tallow, bayberry, saponification of, 251
Tannic acid, influence of, on dextrin, 97
on starch, 93
Tanret's reagent, 892
Tartar, formation of, 105
Taurine, 275, 282, 560, 568, 615
derivatives, 615
formula for, 275, 568
microscopical appearance, 282
preparation of, 281
Taurocholic acid, 275
Teeth, 549, 557
analysis of, 558
composition of, 557, 558
Teichmann's crystals, form of (see Hemin crystals)
test, 349
Tendomucoid, 159, 178, 550, 551
chemical composition of, 178
experiments on, 551
hydrolysis of, 551
preparation of, 551
Test meals, 228
Ewald, 228
retention, 228
water, 228
Testes, 473, 486
Tethelin, 487
Tetramethylenediamine, formula for, 292
Tetranucleotide, 190
Theis and Benedict method for determination of phenols, 400
Thiocyanates in saliva, significance of, 105
ferric chloride test for, 109
Solera's reaction for, 109
Thrombin, 341, 342
Thromboplastin, 342
Thunberg tube, illustration of, 62
Thymine in nucleic acids, 191, 193, 199
Thymovidin, 448
Thymus, 473, 488
histone, 159
nucleic acid, 189, 196
preparation of, 196
tests on, 196
Thyreoglobulin, 479, 482
Thyroid gland, 473, 479
experiments on, 482
hormone, 481
products, determination of the activity of 482
tissue, Kendall's method of determination of iodine in, 483
Thyroxin, 46, 480–482
crystals of, 481
formulas for, 481, 482
Tincture of iodine, preparation of, 893
Tisdall's modification of Folin-Denis method for phenols in urine, 768
Tissue, adipose, experiments on, 250
analysis, 356
connective, 549, **550**
white fibrous, 550
composition of, 550
experiments on, 551
yellow elastic, 552
composition of, 553
experiments on, 553
epithelial, 549
experiments on, 550
muscular, 559
experiments on, 568
nervous, 575
experiments on, 578
osseous, 555
experiments on, 556
Tissue débris in urinary sediments, 697
Titratable alkali of blood, determination of, 440
o-Tolidin test for blood, 239, 321, 348, 652
Tollen's reaction for conjugate glycuronates, 662
arabinose, 86
galactose, 85, 666
pentoses in urine, 664
Töpfer's method for quantitative analysis of gastric juice, 242
Töpfer's reagent, as indicator, 223, 224, 243
preparation of, 243, 893
Total fixed base, Fiske's method for determination of, 783
nitrogen, of urine, quantitative determination of, 711
Total solids, of milk, quantitative determination of, 546
of urine, quantitative determination of, 710

Total phosphorus of urine, quantitative determination of, 776
sulphur of urine, quantitative determination of, 772, 774
in biological material, determination of, 407
Traube stalagmometer, 22
illustration of, 22
Trichloracetic acid, precipitation of protein by, 168, 185
Triketohydrindene hydrate (ninhydrin) reaction, 166
formula for, 124
Trimethyl-oxyethyl-ammonium hydroxide (see Choline)
Trioses, 66
Tripeptides, 116, 160
Triple phosphate, 571, 633, 681
crystalline form of, 633
formation of, 633
Trisaccharides, 67, 91
Trommer's test, 75
Trypsin (see also Pancreatic protease) 46, 57, **256,** 259, 260
action of, upon proteins, 256, 259
experiments on, 260
in stomach, 206, 216, 220, 237
determination of, 237
nature of, 256
quantitative determination of, 237, 261
Trypsinogen, 47, 256
activation of, 47, 257
Tryptic digestion, 256, 259, 260
and peptic activity, reversibility of, 49
influence of bile on, 261
most favorable reaction for, 260
temperature for, 261
products of, 256, 259
Tryptic proteolysis, 256, 259
Tryptophane, 115, 118, 119, **153**
bromine water test for, 260
color reactions for, 154, 260
colorimetric determination of, 154
crystals, 154
formula for, 153
group in the protein molecule, 164
Hopkins-Cole reaction for, 163
mercury compound of, preparation of, 260
occurrence of, as a decomposition product of protein, 115, 118, 119, 153, 256, 260
occurrence of, as an end-product of pancreatic digestion, 256, 260
Tyndall effect, 2
Tyramine, formula for, 292
Tyrosinase, 45
Tyrosine, 45, 115, 118, 120, **148,** 686
crystalline form of, 148
experiments on, 150
in urinary sediments, 686
microscopical examination of, 150
Tyrosine-sulphuric acid, 150

v. Udránsky's test for bile acids, 280, 655
Uffelmann's reagent, preparation of, 239, 893
reaction for lactic acid, 239
Ultramicroscope, 2
Ultraviolet lamps, 827
light, antirachitic activation of food stuffs, 820
Ultraviolet light, destructive action on enzymes, 48
influence of, in rickets, 829
influence of, on enzymes, 48
irradiation, 824
experiments on, 828, **829**
physiological relationships of, 828
radiation (see ultraviolet light), 286, **824**
sources of, 826
Universal respiration calorimeter, 511
Unknown substances in urine, 674
Unorganized ferments, 41
sediments in urine, 681
Uracil, 190, 191, 193, 199
Wheeler-Johnson reaction for, 199
Uranium acetate method for determination of total phosphates in urine, 776
solution, preparation of, 893
Urate, ammonium, crystalline form of, Plate VII opposite p. 685
sodium crystalline form of, 685
Urates in urinary sediments, 684
Urea, 333, 334, **370, 595, 719**
blood, determination of by manometric gas analysis, 470
Clark-Collip autoclave method, 372
determination of, in blood, 370
in urine, 719
experiments on, 598
in blood, manometric determination of, 470
Karr's method, 373
Myers aeration method, 374
in urine, manometric determination of, 470
Urea nitrate, 598, 599
oxalate, 598, 600
Urease, 45, 46, 64, 597, 599
crystals of, 46
preparation of, 64
decomposition of urea by, 370, 599, 719
preparation of, 370, 720
quantitative determination of urea by, 370, 719
Uric acid, 192, 193, 197, 198, 333, 334, **377, 600 736, 856**
calculi, 502
endogenous, 601, 856
study of, 856
enol form, 600
exogenous, 601, 856
experiments on, 603, 856
formula for, 600
in blood, 333, 334
in urinary sediments, 684
crystalline form of (Plate VI opposite p. 602), 604, 684
isolation of, from the urine, 603
keto form, 600
metabolism, 855–858
origin of, 601
quantitative determination of, in blood, Benedict's method, 377
Folin's direct method, 378
Folin's isolation method, 380
in urine, microchemical colorimetric methods, 736
Krüger-Schmidt method, 738
reagents (Reagents and Solutions)
Uricase, 45, 192
Uricolytic enzymes, 43, 192

Urinary calculi, 698
scheme for chemical analysis of, 700
Urinary concrements (see Urinary calculi)
Urinary sediments, 680
Urine, 384
acetoacetic acid in, 656, **659**
acetone bodies, 758
acetone in, **656,** 758, 762, 763
acidity of, 584, 705–710
albumin in, 644
determination of, official method, 756
ammonia in, 627, 724
bile in, 654
blood in, 651
calcium in, 634, **784**
Shohl and Pedley's method for determination of, 784
chlorides in, 630, 780
collection of, 590
collection and preservation of, in metabolism tests, 789
complete analysis of, 594, 790
creatinine in, **605,** 732
dextrose in (see Glucose)
diacetic acid in (see Acetoacetic acid)
effect of exercise on the composition of, 585
ethereal sulphates, 609, 771, 774, 775
fructose in, 667
galactose in, 666
general characteristics of, 581–591
globulin in, 647
glucose in, 638, 744
hydrogen ion concentration of, 705–710
as influenced by diet, 875
by acid and alkali, 878, 879
β-hydroxybutyric acid in, 660, 759
indican in, **610,** 765
inorganic physiological constituents of, 627
lactic acid in, 743
lactic acid, determination of, 743
lactose in, 665
levulose in (see Fructose in)
manometric determination of urea in, 470
nitrogen, total, determination of, in, 711
non-blood proteins in, 648
organic physiological constituents of, 592
oxalic acid in, **615,** 769
β-oxybutyric acid in (see α-Hydroxybutyric acid)
pathological constituents of, 637
pentoses in, 663
phenols in, 611, 766
phosphates in, 631, 776
physiological constituents of, 592–636
potassium in, 634, 786
protein in, Folin's gravimetric method for the determination of, 754
proteins in, 643, 754
proteoses in, 648, 649
pus in, 653
quantitative analysis of, 702
reaction of, 584, 705–710
as influenced by diet, 875
by acids and alkalies, 878
secretion of, 581
sulphates in, 628, 770
sulphur in, 770, 772
total fixed base, 783
total nitrogen determination in, 711
Urine, total phosphorus, 779
total sulphur, 772, 774
urea in, **595,** 719
uric acid in, **600,** 736, 856
urobilinogen in, 624, 768
Urobilin, 582, **624**
tests for, 625
Urobilinogen, 624, **768**
Wallace and Diamond's method for determination of, 768
Urochrome, 582, **624,** 673
Urochromogen, 624, **673**
reaction (Weisz) for tuberculosis, 673
Utilization of calcium and phosphorus, influence of carbohydrates on, 288

Valine, 115, 118, 120, **132**
Van den Bergh test, 396
Van Slyke's method for determination of apparatus for alkali reserve, 432
acetone bodies in urine, 758
method for chloride in blood and tissues, 402
Van Slyke and Cullen's method for urea in blood, 374
for carbon dioxide capacity of plasma, 432
for urea in urine, 720
Van Slyke and Fitz, method for alkali reserve, 446
Van Slyke and Neill, manometric methods, 458–470
Van Slyke and Palmer method for organic acids in urine, 706
Van Slyke plasma bicarbonate titration, 437
Vapor pressure, 2, 902
Vegetable amylase, 45, 55
lipase, 45, 58
protease, 58
sucrase, **59,** 267, 271
Vegetable globulins, 158, 173, 174, 175
Vegetable gums, 67
Venous blood, composition of, 499
Viscosimeter, Ostwald, estimation of viscosity by, 17
Ostwald, illustration of, 17
Viscosity, estimation of with Ostwald viscosimeter, 17
Viscosity of colloidal solutions, 17
Vitamin, 803, 804
antineuritic, 803, 804, **811**
antiophthalmic, **803,** 804
antirachitic, 803, 804, **820**
antiscorbutic, 803, 804, **816**
deficiency, 803, 830
influence of, 831
distribution, 804
pellagra-preventing, 803, 804, **815**
reproductive, 803, 804, **819**
Vitamin A, 247, **803,** 804
assay for cod liver oil, 808
biological assays for, 808
color reactions for, 810
demonstration on, 833
determination of, 808
Fearon's color tests for, 811
nature and properties of, 807
Rosenheim-Drummond color tests for, 810
sources of, 804, 806
storage of, 807

Vitamin B, 803, 804, **811**
constant, 814
demonstration on, 831
determination of, 814
nature and properties of, 812
relation of, to metabolism, 814
sources of, **804**, 812
Vitamin C, 803, 804, **816**
demonstration on, 833
detection and estimation of, 818
nature and properties of, 818
sources of, 804, 816
Vitamin D, 248, 803, 804, **820**
antirachitic factor, **804**, 820
demonstration on, 835
relation of to rickets, 820
Shear color reaction for, 824
sources of, 804, 820
Vitamin E, 803, 804, **819**
nature and properties of, 820
sources of, 804, 819
Vitamin X (see vitamin E)
Vitamins, 803, 830
and dietary deficiency, 803
and dietary deficiency, experiments on, 830
distribution of, 804 (table)
in pellagra (see "P–P factor")
Vitellin, 159, 160, 178
Volhard-Arnold method for determination of chlorides, 780
Vulpian reaction for adrenaline, 476

Walker's method for determination of iron in foods, 413
Wallace and Diamond's method for determination of urobilinogen, 768
Water at meals, influence of, 200, 627, 847, 871
Water test meal, 228
Water, influence of, on metabolism, 847, 871
on protein catabolism, 872
Water deficiency, influence of, 847
Water-soluble "B" (see Vitamin B)
Water-soluble "C" (see Vitamin C)
Water starvation vs food starvation, 848
Waxy casts in urinary sediments, 693
Wedge colorimeter, Hellige-Leitz, 360
Myers', 360
Welker's modified method for purine bases, 740
Welker and Marsh method for deproteinizing milk, 548
Welker and Tracy method for deproteinizing urine, 712
Welker-Hektoen-Kretschmer, protein of, 649
Werner hypothesis, 596
Weyl's test for creatinine, 609
Wheeler-Johnson reaction for uracil and cytosine, 199
White fibrous connective tissue, 550
experiments on, 551
White mice as experimental animals, 802
rats, experiments on, 828–849
Whitehorn method for chlorides in blood, 401
Wohlgemuths' method for quantitative determination of amylolytic activity, 263, 325
author's modification of, 325
Wolter's method for determination of iron in urine, 788

Xanthine, 192, 196, 560, 566, 568
bases (see Purine bases)
crystalline form of, 566
formula for, 192, 568
in urinary sediments, 687
isolation of, from meat extract, 574
silver nitrate, 573
crystalline form of, 573
test, 574
tests for, 196
Weidel's reaction for, 197
Xanthophylls, 333, 538
Xanthoproteic reaction, 163
Xanthinoxidase, 45
Xerophthalmia, 803
Xylose, 67, 86
phenylhydrazine reaction on, 87
relative sweetness of, 71
Tollens' reaction on, 87

Yeast, enzymes of, 42, 45
fermentation by, 79
Yeast for nutrition experiments, 831
growth-promoting substance in (see Vitamin B)
influence on growth, 833
nucleic acid of, 190, 195
formula for, 190
preparation of, 195
tests on, 196
nucleoprotein of, 190, 194
preparation of, 194
tests on, 194
water-soluble vitamine in (see Vitamin B)
Yellow elastic connective tissue, 552
experiments on, 553
Youngburg's modification of Van Slyke and Cullen's method for urea in urine, 721

Zein, 114, 118, 120, 158, 176
decomposition of, 118, 120
Zeller's test for melanin, 672
Zymase, classification of, 45
preparation of, 42
Zymo-exciter, 47
Zymogen, 47, 256

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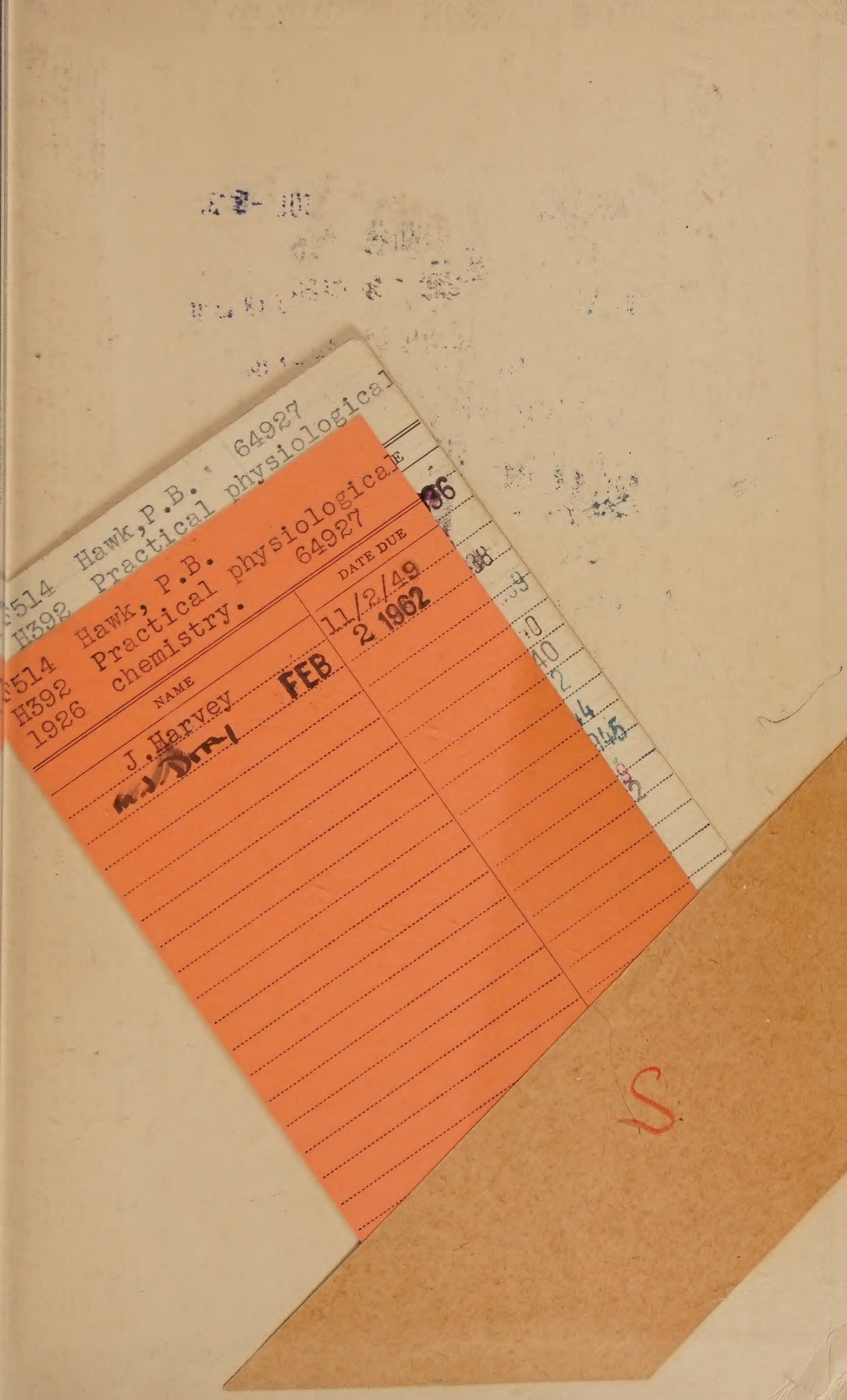
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